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PRELIMINARY INVESTIGATIONS OF THE KINETICS OF NITROGEN TRANSFORMATION AND NITROSAMINE FORMATION IN LAND TREATMENT OF WASTEWATER

S. Jacobson and M. Alexander

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In laboratory experiments, denitrification of nitrate in wastewater proceeded slowly in an acid soil (pH 4.2), but the rate was fast in soils with pH values of 5.5 to 6.8. The rate of denitrification was governed by the carbon source added, with glucose supporting the fastest rate. The rate was somewhat slower with methanol and succinate and was appreciably slower with secondary effluents as the source of supplemental carbon. Charlton loam supported the more rapid denitrification with glucose as a carbon source, but the rate was higher in Windsor sandy loam with sewage as the carbon source. Denitrification in

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20. Abstract (cont'd)

in these soils did not occur at 1^9C, and the rate increased with rising temperatures at 7C, 15C, $21-22$C and 30^oC. Tests of pure cultures of four denitrifying bacteria demonstrated that they needed 0.8 to 2.0 pg of nitrate-N per cell, whereas the requirement was 1.8 to 8.4 pg of nitrate-N per countable denitrifier cell in soil. In addition, the active denitrifying microorganisms in the test soils were characterized.

PREFACE

This report was prepared by Stuart Jacobson and Dr. Martin Alexander
of Cornell University, Department of Agronomy, and was reviewed for
technical aspects by Dr. I.K. Iskandar, T.F. Jenkins and D.C. Leggett
of the Earth Sciences Branch, Research Division, U.S. Army Cold Regions
Research and Engineering Laboratory. The work was funded under Corps
of Engineers Civil Works Project 527021, Work Unit 7070221, Nitrogen
Transformation in Land Treatment of Wastewater. Dr. I.K. Iskandar was
the project officer for monitoring the technical accomplishments during
the course of the study.

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PRELIMINARY INVESTIGATIONS OF THE KINETICS OF NITROGEN TRANSFORMATION AND NITROSAMINE FORMATION IN LAND TREATMENT OF WASTEWATER

by

Stuart Jacobson and Martin Alexander

INTRODUCTION

Wastewater and pollution

The inadvertent pollution of ground and surface water by the introduction of sewage-borne wastes is one of the most urgent problems in the renovation of wastewaters. Unless this problem is understood and its effects kept to an absolute minimum, many natural waters will be polluted.

Chemicals in wastewaters may cause such conditions as the eutrophication of lakes and streams resulting from enrichment nutrients within the wastes, and the contamination of water supplies because of toxic compounds present in the sewage. These problems go beyond the aesthetic realm (as typified by a lake overgrown with algae and larger plants) and extend into the realm of the economic (as when waters are unfit for production of edible fish or for industrial use). Such pollution may even affect public health and well-being, as when water supplies become contaminated and dangerous for human consumption.

Sewage-treatment practices currently in use are adequate for the removal of many organic wastes by converting them to inorganic molecules and for the prevention of the spread of pathogenic microorganisms (33). Yet, these practices do not remove many of the soluble inorganic ions present and will permit the release of these ions in large amounts into natural waters, resulting in eutrophication and contamination (33).

Nitrogen is one of the principal causes of pollution arising from wastewater disposal (23). The sources of the nitrogen are many and include domestic sewage, drainage from fertilized lands, wastes from domestic animals, municipal solid wastes, and industrial and chemical wastes. Quite often, sewage and sewage treatment plants constitute a concentrated source of nitrogen, which must be dealt with; otherwise it may move into waters and become a pollutant.

The concentration of nitrogen in treated sewage leaving a domestic treatment plant will rarely exceed 50 μg/ml, of which approximately 85-90% is in the form of ammonium, about 10-15% is organic, and only a small amount of the nitrogen is present as nitrate (33). Yet even a nitrogen concentration of 5 μg/ml could be deleterious to water supplies.

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Ammonium can stimulate algal blooms, providing that other nutrients such as phosphorus and light are not limiting, and the presence of ammonium may also lead to a reduction of O_2 availability to fish and other animals as the O_2 is consumed by nitrifying bacteria oxidizing the cation to nitrate. Oxygen will also be lost as heterotrophic microorganisms decompose the algae, often leading to unpleasant odors. The state of California has recommended that the nitrogen level in discharges into receiving bodies of water not exceed 2 µg/ml to prevent eutrophication and fish kills (33). Nitrate entering drinking water supplies may also pose difficulties because high nitrate concentrations in ingested waters may lead to cases of methemoglobinemia, a condition where the nitrate is reduced to nitrite by the microflora in the gastrointestinal tract and the nitrite reacts with the hemoglobin in the red blood cells to destroy its capability of transporting oxygen. Methemoglobinemia can be a problem in human infants and ruminants. The U.S. Public Health Service has established a standard of 10 mg of nitrate-N per liter as the maximum concentration recommended for potable water supplies on the basis of studies demonstrating a statistical link between the incidence of methemoglobinemia in infants and nitrate concentrations in drinking waters (33).

The Nitrogen Cycle

Nitrogen, like a few other elements, undergoes a cyclic series of transformations in which the element passes through gaseous and non-gaseous states, both oxidized and reduced. This cycle has been studied for over a century (35), and the steps involved are the following.

$N_2 \rightarrow NH_4^+$. Nitrogen fixation. This step, sometimes called dinitrogen fixation, is an energy-requiring step. It may be carried out by bacteria, either alone or in conjunction with a higher plant, and by blue-green algae. It may also be carried out industrially by man.

N organic $\rightarrow NH_4^+$. Mineralization. This step makes nitrogen available to plants. Mineralization is carried out by many heterotrophs. Under conditions of high pH, the ammonium may be lost as ammonia through volatilization.

$NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$. Nitrification. Nitrification, an energy-yielding step, may be carried out by autotrophic bacteria or possibly by heterotrophic species of bacteria or fungi. For the heterotrophic process, the conversion requires a carbon source (1,35).

$NH_4^+ \rightarrow$ N organic, ammonium assimilation. This step is carried out by many bacteria, fungi, algae and higher plants. The nitrogen is incorporated into protein and nucleic acids.

$NO_3^- \rightarrow NH_4^+ \rightarrow$ N organic. Assimilatory nitrate reduction. Nitrate is assimilated by many bacteria, fungi, algae, and higher plants. This

step requires energy and is also known as immobilization because it renders the nitrogen unavailable to plants and to much of the microflora.

$NO_3^- \rightarrow NH_4^-$. Dissimilatory nitrate reduction. This is an energy-requiring step that may be carried out by some bacteria and fungi.

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Denitrification. Denitrification, or nitrate respiration, is an energy-requiring process that is carried out solely by bacteria. It is the portion of the nitrogen cycle whereby nitrogen returns to its gaseous dinitrogen (N_2) state.

In order to minimize environmental pollution arising from wastewater treatment, the nitrogen must be removed. Since most of the wastewater nitrogen is in the form of ammonia, a plausible way to remove this N from the water is to make use of two steps in the nitrogen cycle, nitrification and denitrification. These two steps may be consecutive in the soil, and proper management of the two steps could ensure against water pollution resulting from nitrogen input.

Incoming ammonium will, by virtue of its positive charge, become fixed on the soil's negatively charged clay micelles. Nitrate, with its negative charge, will simply travel vertically down the soil horizon, into the groundwater and then into drinking supplies or into surface waters (15). The problem of nitrogen in wastewaters can be now studied in terms of ammonium oxidation and nitrate reduction.
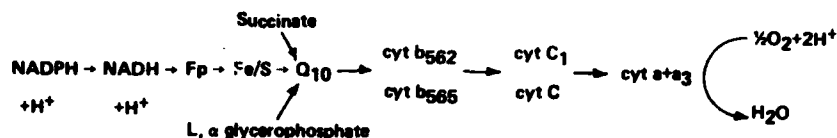
Nitrification and denitrification

Nitrification was first studied a century ago when Schloesing and Muntz (69) discovered that the production of nitrite and nitrate ions from ammonium in sewage percolating through soil could be terminated by the addition of chloroform. Researchers trying to isolate the responsible agents by the common procedures of that time met with no success until Winogradsky (90), in 1890, realized that the oxidation of the ammonium was the energy supply for the bacteria performing the transformation and that they had no requirement for eauced carbon compounds. He saw that the bacteria were able to manufacture cellular constituents by the fixation of carbon dioxide. This was the first time that chemoautotrophic growth was recognized. Since then, nitrification has been studied extensively (35). Though Bergey's Manual of Determinative Bacteriology (18) lists four genera of autotrophic ammonium oxiders (Nitrosomonas, Nitrosococcus, Nitrosospira, and Nitrosolobus) and three genera of nitrite oxidizers (Nitrobacter, Notrococcus, and Nitrospina), only Nitrosomonas and Nitrobacter have been extensively studied (35). Most of the reports in the literature mention these two genera as being responsible for nitrification in nature, though their relative contribution to the process as compared to the other autotrophic genera and to the over 25 reported genera of heteorotrophic nitrifiers is uncertain.

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Nitrifying bacteria reach the highest numbers in surface soils at depths of 0-10 cm, where one usually finds the highest levels of total nitrogen (35), the highest levels of O_2 and the highest cation exchange capacity (15), which would retain the ammonium for the bacteria to utilize it. Studies have shown that populations of Nitrosomonas and Nitrobacter may increase from several hundred to several million per gram of soil upon the addition of ammonium to the soil (55). Researchers generally feel that most of the nitrate generated in soil results from autotrophic nitrification rather than heterotrophic nitrification (35). 2-chloro-6-(trichloromethyl)pyridine is a chemical that selectively inhibits autotrophic nitrification in culture (31). When it is added to soils, the compound markedly reduces the rate of nitrification and thus it appears that heterotrophic nitrification is not significant (31). However, a few experts have suggested that heterotrophic nitrification may be significant in soils (81), acid soils (82), and muck soils (Histosols) (35).

Denitrification as a biological process mediated by microorganisms was first studied in 1868 (35). Until then, it was thought to be a purely chemical process. Gayon and Dupetit (38) observed in 1886 that the disappearance of nitrate and nitrite with the concurrent production of nitrous oxide and nitrogen gas in anaerobic sand columns perfused with nitrified effluent was actually a biological process. In 1895, it was proposed (35) that the denitrifying bacteria in soil were derived from manure, and thought that they should be eliminated because great losses of soil nitrogen were feared. This misconception as to the vector for denitrifiers was cleared up in 1907 when Deherain (25) noted that the denitrifying bacteria were already present in the soil and were merely stimulated by the nutrients in the manure. In 1902, Weissenberg (88) stated that the reduction of nitrite and nitrate to gaseous products was performed by typical aerobic bacteria that simply switched to using the oxygen present in nitrate when there is no molecular oxygen available. Though Weissenberg was correct in the first part of his assertion (i.e. that the process was affected by aerobes), his understanding of the role of nitrate in denitrification was incorrect. Nitrate itself serves as an electron acceptor. The electron transport chain to nitrate has been extensively studied (44), and a representation of the aerobic and anaerobic electron transport chains in Paracoccus denitrificans may be seen in Figure 1.

Denitrification is brought about by the same respiratory electron transport chain (with slight modifications) present in many aerobic bacteria, yet much of the current literature incorrectly states that facultative anaerobes are responsible for denitrification (35). Facultative anaerobes utilize the full cytochrome system when O_2 is available as the terminal electron acceptor, but they will use various organic compounds as electron acceptors when oxygen is not available; i.e. they will carry out fermentation. Strict aerobes and denitrifiers cannot ferment. However, facultative anaerobes are capable of using nitrate as
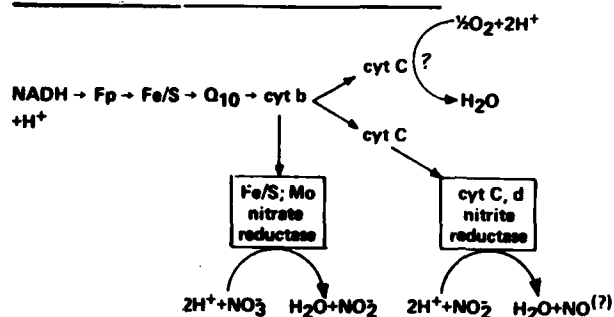
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a.) AEROBIC ELECTRON TRANSPORT

Succinate

$NADPH \rightarrow NADH \rightarrow Fp \rightarrow Fe/S \rightarrow Q_{10} \rightarrow$ cyt b_{562} $\rightarrow$ cyt C_1 $\rightarrow$ cyt $a+a_3$ $\rightarrow$ ½O_2+2H^+

$+H^+$ $+H^+$ cyt b_{565} cyt C $\rightarrow H_2O$

L, α glycerophosphate

b.) ANAEROBIC ELECTRON TRANSPORT

½O_2+2H^+

cyt C (?)

$NADH \rightarrow Fp \rightarrow Fe/S \rightarrow Q_{10} \rightarrow$ cyt b $\rightarrow H_2O$

$+H^+$ cyt C

| Fe/S; Mo nitrate reductase | cyt C, d nitrite reductase |

$2H^+$+NO_3^- H_2O+NO_2^- $2H^+$+NO_2^- H_2O+$NO^{(?)}$

Figure 1.   Electron transport chains in <u>Paracoccus denitrificans</u>
grown aerobically (a) and anaerobically with nitrate
(b). Abreviations: Fp, flavoprotein; Fe/S, iron-
sulfur protein; Q_{10}, ubiquinone; cyt, cytochrome.
cyt b_{562}, cyt b_{565}, cyt C_1 and cyt C represent b and c
type cytochromes differentiated by their spectral
properties. During anaerobic growth with nitrate, in-
creased cytochrome c is synthesized, but its signifi-
cance at this point is uncertain (44).

an electron acceptor, but they only reduce it to nitrite in the process
of dissimilatory nitrate reduction (1). There is some confusion in
the literature as to which bacteria are denitrifiers. Bergey's <u>Manual</u>
<u>of determinative bacteriology</u> (18) is unclear on the subject, the
authors often attributing to a bacterium the ability to reduce nitrate
but neglecting to note whether the reduction goes past nitrite.

Many genera of bacteria have been shown to denitrify, including:
<u>Acetomonas</u>, <u>Alcaligenes</u>, <u>Bacillus</u>, , <u>Corynebacterium</u>, <u>Flavobacterium</u>,

<u>Halobacterium</u>, <u>Listeria</u>, <u>Paracoccus</u>, <u>Pseudomonas</u> and <u>Thiobacillus</u> (1, 35), but the relative contribution of these genera to the process in the environment is uncertain. Some studies (37, 78, 83) suggest that the genera <u>Pseudomonas</u> and <u>Alcaligenes</u> (including <u>Achromobacter</u>, which until 1974 was a separate genus) are the most common genera of denitrifiers in soil. On the other hand, denitrifying members of the genus <u>Bacillus</u> have also been found in high numbers (60). Another researcher (91) has found a preponderance of <u>Bacillus</u> types at low nitrate levels in soil, and after the soil was enriched with nitrate and incubated anaerobically, this investigator found greater numbers of gram-negative denitrifiers. He concluded that the soil environment was more conducive to the development of gram-negative types. One also must consider, especially in soil, that spore-forming bacteria, though present in apparently high numbers, may be in the resting stage and may have little effect on processes taking place around them.

Environmental effects on nitrification and denitrification

As alluded to above, in any biological process the rate of a process depends upon the environmental conditions which are responsible for the selection of the organisms to carry out the process. In a process like nitrification, where there presumably is a very low species diversity, tolerance to environmental change is low. In denitrification, the diversity of types of active organisms would permit the process to withstand greater environmental fluctuations. There are some generalizations that can be made as to the influences that various environmental parameters may have on the two processes.

Nitrification requires aeration, an energy source (ammonium for autotrophs and a carbon source for heterotrophs), and a neutral to slightly alkaline pH. It is inhibited by pH extremes, temperature extremes, high substrate levels, end-product accumulation (this effect is pH dependent), and lack of oxygen (35). Evidence indicates that nitrification may also be inhibited by high sulfur dioxide concentrations in the air (52).

Nitrification rates are related to temperature. Autotrophic nitrification is optimal at temperatures ranging from 25-35°C (33), with a cessation of the process as the temperature approaches 40°C. Heterotrophic nitrification has been reported to occur at temperatures above the range for autotrophic nitrification (32) and even at 55°C (48), but the significance of this phenomenon probably is limited to few environments. It has also been suggested to occur in desert soils (32) and during solid waste composting (48) but not during liquid waste composting (35).

Temperature has less influence on denitrification than on nitrification; this is a result of the greater species diversity among the denitrifiers than among the nitrifiers. There is a wider temperature

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range for denitrification than for nitrification, from 5-85°C (60), with an optimum at 65°C. Some workers have reported lower temperature optima for denitrification, ranging from 35-45°C (16,73), but it is thought that these investigators did not wait long enough to permit a species succession that would allow thermophilic species of Bacillus, which were originally present in low numbers, to become dominant (35). There have also been reports indicating that denitrification can take place at temperatures approaching 0°C (72).

The pH of the environment has a considerable effect on nitrification, which is to be expected because of the low species diversity and the fastidiousness of the organisms involved. The pH optimum for the process lies in the neutral to low alkaline range (35). At high pH, values, ammonium is in the ammonia (NH_3) form, which may spontaneously volatilize. Ammonia is toxic to Nitrosomonas and Nitrobacter (4); nitrite at low pH values is in the nitrous acid (HNO_2) form which is toxic to Nitrobacter (4). The pH effects on both steps of the nitrification process are linked with the ammonium and nitrite concentrations and are summarized in Figure 2 (4).
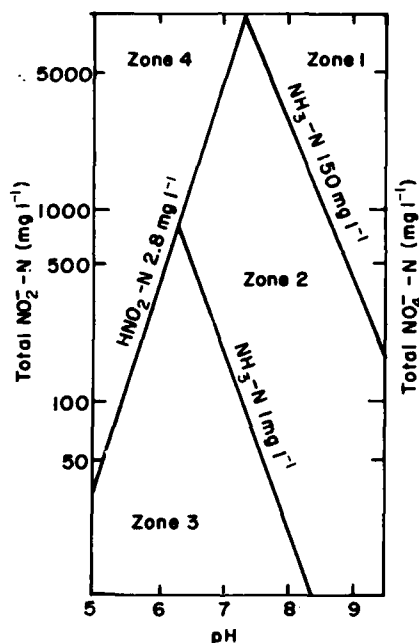

Figure 2.   Nitrification tolerance graph.

Zone 1:   Inhibition of Nitrosomonas and Nitrobacter
by NH_3

Zone 2:   Inhibition of Nitrobacter by NH_3

Zone 3:   Complete nitrification

Zone 4:   Inhibition of Nitrobacter by HNO_2 (4)

Four types of nitrification patterns have been characterized
(57) (Fig. 3-5). In soils of slightly alkaline pH (e.g. pH 7.9), there
is an initial rapid ammonium oxidation coinciding with a buildup of
nitrite. Nitrite accumulation continues until most of the ammonium
has disappeared, and then the nitrite oxidation proceeds but only *after*
a long lag phase. In a soil of pH 6.4, by contrast, there is a quick
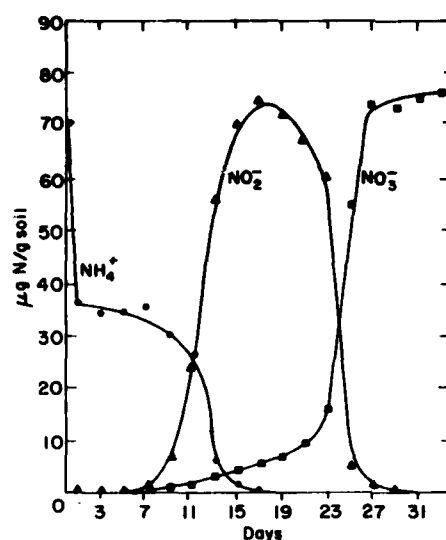oxidation of both the ammonium and the nitrite formed from it, so that

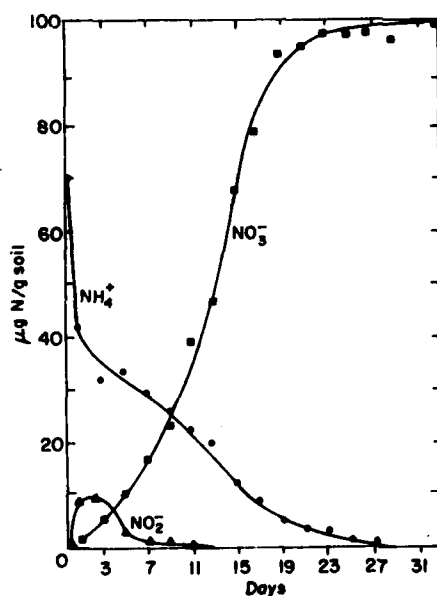Figure 3. Nitrification pattern in a soil of pH 7.9 (57).

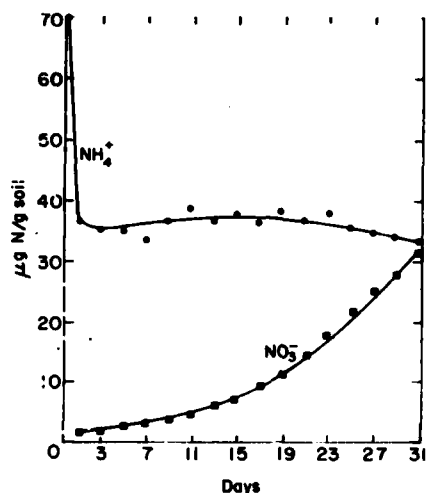Figure 4. Nitrification pattern in a soil of pH 6.5 (57).

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Figure 5. Nitrification pattern in a soil of pH 5.4 (57).

no real nitrite buildup occurs. At a pH of 5.8, the same nitrification
pattern as at pH 6.4 took place, except at a slower rate. At a slightly
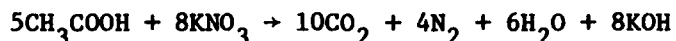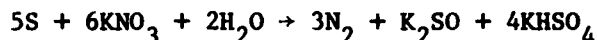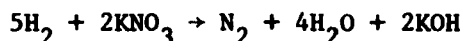lower pH value (5.1), the process did not occur.

Heterotrophic nitrification has been shown to occur at alkaline
pH values (pH 8.0-9) (83). It has also been suggested to occur in soils
that have a pH of 4.5 or less (35), with organic nitrogen being con-
verted to nitrate. The addition of chemicals stimulatory to autotrophic
nitrification, such as lime or ammonium salts, has only had inhibitory
effects on the process (35, 87), implying heterotrophic activity.

The denitrification process is less sensitive to pH "extremes"
than nitrification. Reports indicate that denitrification will occur
at pH values ranging from 3.5 (60) to 11.2 (67), with most denitrifiers
having an optimum pH for growth in the range of pH 5.0-9.0 (35).
Denitrification itself has a pH optimum in the neutral to slightly
alkaline range (35), like nitrification.

The pH, temperature and degree of aeration all exert an influence
on the composition of end products in the denitrification process.
Lower pH values, lower temperatures, and higher oxygen tensions all
contribute to higher proportions of nitrous oxide being released (27,
60,89). It is thought that the effects of temperature and pH on the
end product composition are due to the fact that these factors play a
major role in the selection of the dominant bacteria for the process

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(20,35). It has been shown (20) that greater concentrations of dinitrogen compared to nitrous oxide release can be achieved in low pH soils by having longer incubation periods. Nitric oxide has also been detected in soils where denitrification is taking place at low temperatures and at low pH values (60,89), but in the latter case the reduction of the nitrate to the nitric oxide is considered to be nonbiological, especially since the same amount of nitric oxide was evolved in the sterilized control.

Denitrification requires an energy source. The organism receives only 60% of the energy from the electron donor as when oxygen is used as the terminal electron acceptor (65). With the exception of Paracoccus denitrificans (which is able to utilize H_2 as an electron donor) and Thiobacillus denitrificans (which can use elemental sulfur and some reduced sulfur compounds), the denitrifying bacteria make use of reduced carbon compounds for energy. The reactions are:

$$5H_2 + 2KNO_3 \rightarrow N_2 + 4H_2O + 2KOH$$

$$5S + 6KNO_3 + 2H_2O \rightarrow 3N_2 + K_2SO + 4KHSO_4$$

$$5CH_3COOH + 8KNO_3 \rightarrow 10CO_2 + 4N_2 + 6H_2O + 8KOH$$

The most efficient carbon:nitrogen ratio for nitrate removal from sewage is about 2.5:1 (33). The same ratio applies to soil, implying that utilizable carbon is limiting in both soil and sewage (35). Adjusting the C:N ratio to higher levels has no significant effect on the denitrification rate, but very high ratios will promote fungal growth if air is present and will promote assimilatory nitrate reduction when O_2 is absent (33).

Most of the native soil organic matter is largely unavailable to denitrifying bacteria. The supply of readily decomposable organic matter is a critical factor for denitrification. Bremner and Shaw (16) found that the effect of organic matter on denitrification in water-logged soils was related to the resistance of the organic substances to decomposition. Compounds that were easily degradable, like simple six-carbon sugars, had a great effect on denitrification, while more recalcitrant substances like lignin and sawdust had little effect. They also determined that the stimulation of denitrification by wheat or oat straw was considerably lessened when the straws were leached with water or allowed to decompose slightly before being added to the soil. In a later report, Burford and Bremner (19) found a relationship between a soil's capacity to denitrify and its content of water-soluble organic carbon.

Denitrification requires anaerobiosis and thus is inhibited by the presence of molecular oxygen. In most environments that may appear

to be well aerated, anaerobic microsites exist (93). This is especially true in a heterogenous environment such as soil, where an anaerobic zone can be adjacent to an aerobic site, and nitrogen losses thus can occur at the same time that nitrification is proceeding. This has been shown in soils (75), oxidation ditches (59), activated sludge tanks (92), and incubated chambers which contain growing plants (76). The anaerobic microsites are often too small to detect with an oxygen probe or electrode. Their creation is dependent on three factors (93):

1. The oxygen consumption rate of the surrounding microflora and microfauna. This factor is also related to the amount of available carbon, pH, and the temperature.

2. The rate and amount of oxygen diffusion to the microsite. This factor may also be related to the temperature.

3. The geometry of the microsite. The structure and dimensions of the microsite can play a role in determining the rate and amount of oxygen diffusion to the site.

Many reports have been published describing anaerobic and putative aerobic denitrification, and Painter (63) in his review of the field concluded that many of the studies reporting the latter did not have sufficient information concerning the soluble oxygen concentration. The most important parameter in the question of aerobic denitrification is the soluble oxygen concentration at the microsite level, not in the general surrounding environment.

Because of the difficulties involved in determining soluble oxygen concentrations in a soil sample, many researchers measure the E_h (oxidation-reduction potential) at the site. Nitrate is reduced at E_h potentials ranging from 300-350 mV in the soil and at ranges of 250-350 mV in pure cultures of various denitrifiers (33). The E_h of a system may or may not be related to the amount of oxygen present. Recently, researchers have shown that the growth rate of an obligate anaerobe is not affected by changes in the E_h as long as there is no oxygen present (61). The E_h of a system may not affect the enzymes involved in denitrification, provided that the oxygen tension is low enough. In light of these recent findings, the literature dealing with denitrification and E_h potentials should be reconsidered, and data on soluble oxygen should be examined.

Oxygen is a preferred electron acceptor to nitrate for the denitrifiers. This has been shown in pure culture (71), ocean waters (68), and sewage (24). Denitrification may start, when oxygen is limiting, at concentrations ranging from 0.1-0.2 mg/l (93), and the process has been reported to occur at oxygen levels as high as 0.7 mg/l in the ocean (40).

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The texture of a soil has an effect on the nitrification and den-
itrification taking place within it. Sandy, well-aerated and well-drained
soils provide better conditions for nitrification to occur. Paradoxically,
sandy, well-drained soils also allow for rapid nitrate leaching and are
not favorable for denitrification (15). It has also been shown that, in
soils containing an argillitic horizon of high silicate clays (which would
tend to have poor drainage), the majority of nitrate tended to be
denitrified rather than leached (55).

Nitrification and denitrification in wastewater

In practice, denitrification in sewage can be stimulated by the
addition of such substances as methanol, molasses, humus, cellulose,
hydrogen, sugars from bakery wastes, methane, elemental sulfur, and
by-passed primary effluent that was blended with secondary effluent
(35). Methanol is a cheap chemical additive and provides little carbon
for assimilatory purposes (58). Methane and humus are not as rapidly
oxidized as methanol. The sugars tend to promote the assimilation of
nitrate and must be added with care (33). Sulfur has its drawbacks for
the purpose of removing nitrate from sewage, because acid production
in its oxidation requires careful monitoring of the pH and may necessi-
tate the addition of buffers, such as lime (33).

The spreading of effluents on land has achieved some degree of
success in renovating wastewater, especially in warm and dry areas
where there is a lot of open land. One project in Arizona has been
able to renovate wastewater economically by spreading it on the soil
(14). The Arizona process requires dry and wet cycles. The dry periods
after the infiltration of the wastewater are necessary for the soil to
be aerated to permit nitrification to occur. The wet periods allow
anaerobic conditions to develop in the soil to encourage denitrification.
Sometimes, the carbon remaining after nitrification has taken place may
not be present in adequate concentrations for denitrification to progress
to completion. The problem arising from the lack of carbon available to
the denitrifiers has been remedied by adding primary effuent to the
mixture (53) or by growing plants on the soil that will receive the
wastewater (8). This work, along with work done in other climates, has
shown the stimulatory effects that growing plants have on denitrifica-
tion. The plant roots excrete organic materials into the rhizosphere.
These chemicals provide a constant carbon source that is available to
both the denitrifying populations and the oxygen utilizers; the latter
would help to maintain a low oxygen tension in the soil (35).

The wet and dry periods mentioned in the processes above are also
employed to prevent the clogging of the soil pores by sludge particles.
The clogging of pores would have detrimental effects on the processes
of nitrification and denitrification in that it would 1) promote anoxic
conditions in the soil at times when oxygen is required for nitrification

to occur and 2) inhibit the vertical infiltration of new liquids into the soil. Both of these conditions would defeat the purpose of land spreading of wastewater in that the hinderance to nitrification caused by anaerobiosis would reduce the efficiency of a sewage treatment project. Also, the lack of vertical flow of liquids into the soil would cause horizontal runoff, the constituents of which would presumably be transported to a body of natural water, leading to nutrient enrichment which is just what a sewage treatment project intends to prevent.

The wet and dry periods are also important, because when a soil is dried and then wetted again, organic matter is made available to the indigenous microflora (11). A treatment involving extremes in wet and dry cycles has led to extensive losses of indigenous soil organic nitrogen through mineralization-nitrification-denitrification and also extensive losses of added nitrate (35).

Nitritification and denitritification are processes that involve the oxidation of ammonium to nitrite and the reduction of nitrite to nitrogen gas. Researchers (67) have suggested employing nitritification and denitritification instead of nitrification and denitrification in treating wastewaters. Utilization of these processes would have some advantage over the complete oxidation-reduction steps occurring in nitrification-denitrification. First, it would take less time. Second, it would require less oxygen for the oxidation, and less carbon to reduce the nitrite. Third, it would omit the possibility of ammonium toxicity to Nitrobacter, since this organism would play no part in the process (35). Finally, nitrite could be removed chemically and converted to molecular nitrogen by acidification and the subsequent addition of urea (67).

The evolution of air pollutants during nitrification and denitrification

Nitrous oxide has been reported to be released not only during denitrification but also by Nitrosomonas europaea (94) from an intermediate in the oxidation of ammonium and by fungi (13) during nitrite reduction. Nitrous oxide can destroy the ozone layer in the upper atmosphere via this series of reactions:

(1) $O_3 \rightarrow O_2 + O$

(2) $O_2 + 2O \rightarrow 2O_3$ (ozone formation)

(3) $N_2O + O \rightarrow 2NO$

(4) $NO + O_3 \rightarrow NO_2 + O_2$ (1)

The ozone layer protects the earth from harmful ultraviolet radiation by filtering out this UV light (27). Increased exposure to ultraviolet

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radiation can lead to a higher rate of skin cancer and will have an adverse effect on plant growth (27). Thus, the release of N_2O during nitrification and denitrification may have a profound effect on the fate of the ozone layer and should be kept to a minimum.

The formation of nitrosamines during nitrification and denitrification

During nitrification and denitrification, and especially during the combined nitritification-denitrification process, there may be relatively large accumulations of nitrite. It has been shown that nitrite can react with a secondary amine to form a nitrosamine (6,7,56) by the general reaction:

$$R' - \overset{\overset{\displaystyle R}{\displaystyle |}}{N} - H \ + \ NO_2^- \ \rightarrow \ R' - \overset{\overset{\displaystyle R}{\displaystyle |}}{N} - N = O$$
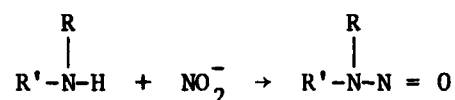
Many of the nitrosamines are carcinogenic, mutagenic, and teratogenic, and some are nonbiodegradable (1,77). Also, some will move vertically through the soil profile (1), and the only way that they can be destroyed is by cleavage by ultraviolet radiation. Secondary amines are ubiquitous in the environment, their sources being (directly or indirectly) plant tissues, animal tissue, pesticides, animal wastes and surfactants (1). Dimethylnitrosamine has been produced in the laboratory in samples of soil (7,56), sewage (6), and lakewater treated with secondary amines and nitrite. Though researchers have yet to find nitrosamines being formed as a result of nitrification and/or denitrification of waste- waters (84), the possibility of nitrosamine formation remains real.

Considerations

It is clear that before a project involving land spreading of wastewater is to be embarked upon, many factors must be examined. These factors include:

1. The soil's ability to nitrify and to denitrify.

2. The soil's ability to absorb influent waters.

3. The proximity of the soil to local water supplies.

4. The possibility of nitrosamine formation in the soil during nitrification and/or denitrification.

Before proceeding with a land spreading operation, these factors must be taken into account. In light of these concerns, this study was undertaken. The aim of the study was:

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1. To determine the kinetics of nitrate loss and nitrate accumulation and the disappearance of nitrate in several soils as related to soil pH, temperature, exogenous carbon source, and counts of denitrifiers.

2. To identify the prominent and active denitrifiers in a soil in which denitrification is taking place.

3. To note the possible formation of dimethylnitrosamine during denitrification in soil.

MATERIALS AND METHODS

Soils. In the experiments studying denitrification, four soils
were used; Lordstown channery silt loam (pH 4.2, 4.5% organic matter),
Niagara silt loam (pH 6.8, 6.2% organic matter), Charlton loam (pH
6.3, 4.9% organic matter) and Windsor sandy loam (pH 5.3, 4.4% organic
matter). The first two soils were gathered in the Ithaca, New York,
area, and the latter two soils were supplied by I.K. Iskandar of
CRREL. For the isolation of denitrifiers, Hudson silty clay loam (pH
6.8, 5.1% organic matter) from the Ithaca area was used. The Ap horizon
of each soil was taken.

In the kinetics studies, the soils were stored at room temperature
in the dark. Prior to use, they were air dried and sieved through a
10-mm sieve. At the beginning of each experiment, the soils were
brought to field capacity with a nitrate-carbon source solution to
bring the nitrate concentration to 100 ppm NO_3^--N. Unless otherwise
noted, the soils were treated with glucose as the carbon source, other
carbon sources being succinate, methanol (at concentrations of 500 ppm
glucose-C, primary and secondary effluents) and endogenous soil organic
matter.

In the isolation of denitrifiers, freshly dug, undried soil was
used, and a nitrate-glucose solution was added to one set of samples
and an ammonium-glucose solution was added to another set of samples to
bring the nitrogen concentration up to 100 ppm NO_3^--N or NH_4^+-N and to
achieve a carbon concentration of 500 ppm glucose-C. The moisture
content of the soil was brought up to 40% (w/w) with the nutrient
solution.

In the initial kinetic studies with the Niagara and the Lordstown
silt loams and in the experiments isolating denitrifiers from the Hudson
silty clay loam, the soils were moistened, mixed, and then put into
250-ml Erlenmeyer flasks. These flasks were stoppered with no. 4
rubber stoppers through which glass tubing was placed, and prepurified
N_2 (99.996% N_2) was passed through the flasks for not less than two
minutes. The flasks were then incubated at the desired temperature
in the dark.

In studies of the Charlton loam and the Windsor sandy loam and
subsequent studies of the Lordstown and Niagara silt loams, purified
N_2 was constantly passed over the wetted and mixed soil, which was
contained in 200x25-mm filter tubes. The gas was passed at a flow
rate of 21 ml/min, first through the water trap, then through a mani-
fold, and finally over the soil samples. At least 25 to 35 g of soil
was used in every sample. The temperature was maintained by using a

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constant temperature room or by immersing the filter tubes to a depth greater than the top of the soil in a Lauda RC-20 refrigerated water bath maintained at the proper temperature with a Lauda B-1 heating pump.

Analytical Methods. Nitrite and nitrate determination were made on samples of soil and cultures at regular intervals. In the initial experiments, the anions were extracted from the soil with 0.2% $Ca(OH)_2$. In the latter experiments, the soils were extracted with distilled water. The extractions were performed by the intermittent shaking of the soil-liquid mixture (which contained 9 parts extracting solution: 1 part soil) for 30 min. The mixture received 0.5 g Darco carbon per 20 g mixture to decolorize the filtrate and was filtered through Whatman no. 42 filter paper.

Nitrite determinations were performed by the use of the sulfanillic acid and N-(1-naphthyl) ethylenediamine method (10). The initial nitrate determinations were made by the chromotropic acid method (10), but the latter determinations employed the Orion nitrate electrode. For the latter nitrate determinations, separate soil extracts were made using an extracting solution containing: $Al_2(SO_4)_3 \cdot 18H_2O$, 16.66 g; H_3BO_3, 1.25 g; Ag_2SO_4, 4.67 g; NH_2SO_3H, 2.43 g; distilled H_2O, 1 liter. This solution, which was brought to pH 3.0, was recommended by the Orion Co. (62). The nitrate in the soil extracting solution mixture, which contained 4 parts extracting solution: 1 part soil, was measured directly by the electrode.

Sewage. In the studies using municipal effluents, the primary and secondary effluents were obtained from the Ithaca, New York, sewage treatment plant. The soils were wetted to field capacity with the effluent enriched with KNO_3 to a final concentration of 100 ppm NO_3-N. Organic matter concentrations in sewage were not determined.

Nitrate loss per cell. In the experiments performed to assess cell numbers per amount of nitrate lost, denitrifying cultures of Pseudomonas stutzeri (obtained from the culture collection of the Laboratory of Soil Microbiology at Cornell University), Flavobacterium sp., Pseudomonas fluorescens, and Pseudomonas sp. (supplied by J.M. Tiedje of Michigan State University) were first grown on New Brunswick Industries Model G40 rotators (at 140 rpm), at 28°C to a point of turbidity in Difco nitrate broth. Samples of the turbid cultures were diluted 1:1000 in fresh nitrate broth and transferred to tightly sealed screw cap (36.5 ml/tube), and incubated at 28°C, undisturbed. At regular intervals, nitrate and nitrite were determined, and plate counts were made on the samples using Difco nitrate agar. When nitrate began to rapidly disappear, at the time when the appearance of gas bubbles was evident, active denitrification was considered to have started. From this point, the growth rate and the rate of nitrate loss were used to calculate the number of cells that can grow per unit of nitrate nitrogen.

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Denitrifier counts. For the counting of denitrifiers in soil samples, three media were tested: Difco nitrate broth as used by Focht and Joseph (34); a modification of the sodium casinate medium of Fred and Waksman (36) to which KNO_3 was added to a final concentration of 0.1%; and a soil extract medium composed of 1.0 g glucose, 0.70 g KNO_3, 0.50 g K_2HPO_4, 100 ml soil extract, and 900 ml of distilled water. The soil extract was prepared by adding distilled water to the Niagara silt loam to a ratio of 2:1 (v/w), autoclaving for 2.0 h at 115 psi and 121°C, and then using the supernatant fluid after centrifuging for 15 min at 4080 x g. Denitrifying activity was noted in the last two media in the manner used by Focht and Joseph (36); for this purpose, inverted Durham tubes were included to note gas evolution.

Isolation of denitrifiers. In the experiments performed to isolate denitrifying bacteria, three media were tested. Focht and Joseph's media, sodium casinate medium, and the soil extract medium. All were solidified with 1.5% agar, and dilutions of the same soil sample were plated anaerobically in desiccator jars using the GasPak system (Becton Dickinson Corp.) and steel wool coated with acidfied $CuSO_4$ (64) to maintain anaerobiosis.

Serial dilutions from samples of the ammonium and nitrate treatments of the Hudson silty clay loam were made and plated on the nitrate agar (five plates per dilution) after 0, 15, 27, and 29 h of incubation at 28°C. Plates were incubated anaerobically, and MPN counts for denitrifiers were taken at those times and also at 51, 63, and 75 h. From the plates at the 10^{-6} dilution (from the zero-time sample) and from the plates at the 10^{-7} dilution at each subsequent sampling time, 100 colonies were selected from the three of the five plates with the highest colony count and clearest resolution. These were picked and restreaked in duplicate on nitrate agar, the plates being incubated in air. The colonies that then grew were tested for cytochromes (26), the presence of which is essential for denitrification to take place. The cytochrome-positive isolates were purified by restreaking and staining and tested for denitrifying activity using the method that was used to count denitrifiers in soil. All those isolates that were determined to be denitrifiers were then identified using the method of Skerman (70) or Gordon (41) or by means of the Oxi-ferm tubes (LaRoche Diagnostics).

Nitrosamines. In the studies of nitrosamine formation, the conditions were the same as for the denitrification experiments, except that 7.0 ppm N as dimethylamine was also added to the soil. The soil was extracted with 0.2% $Ca(OH)_2$ added at a ratio of 1.5:1 (v/w). Spontaneous nitrosation is not likely in an alkaline extract. This extract was then extracted three times by shaking with methylene chloride to remove possible dimethylnitrosamine from the aqueous solution. Whatever water remained was removed by adding anhydrous Na_2SO_4. The Na_2SO_4-water precipitate was then shaken with more methylene chloride and

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filtered through Whatman no. 42 filter papers. Ethyl acetate (2.0 ml) was added to reduce the volatilization of any nitrosamine and the liquid was then reduced in volume to 2.0 ml at 65°C in a Kuderna Danish evaporator. The sample was then analyzed for the presence of dimethylnitrosamine employing the method used by Mills (56).

The frequency of sampling ranged from 3-h intervals during the anticipated logarithmic phase (of denitrification) to 12-h intervals when the rate of denitrification was expected to be slow. Treatments and analyses were conducted in duplicate.

RESULTS

Denitrification Kinetics

Denitrification rates were measured in the Charlton loam, Windsor
sandy loam, Niagara silt loam, Lordstown channery silt loam, Hudson
silty clay loam and in primary effluent. The rates were measured in
relation to the soil temperature, the carbon source for the reaction
and the soil pH.

Temperature effects on denitrification

The effect of soil temperature on denitrification was measured
in the Charlton loam and the Windsor sandy loam. The soils were in-
cubated at 1°, 7°, 15°, room temperature (21-22°) and 30°C with glucose
(at 500 ppm C) as the carbon source. The Windsor sandy loam was also
incubated at 24°C. In both soils there was no nitrate lost after 7
days at 1°C. At this point, the experiments were terminated. At 7°C,
there was a slow denitrification rate in both soils (Fig. 6), with the
Windsor sandy loam attaining a slightly higher rate of nitrate loss once
the logarithmic phase is established. The rates increased considerably
(Fig. 7 and 8) in both soils at higher temperatures, with the Windsor
sandy loam continuing to have a slightly higher denitrification rate
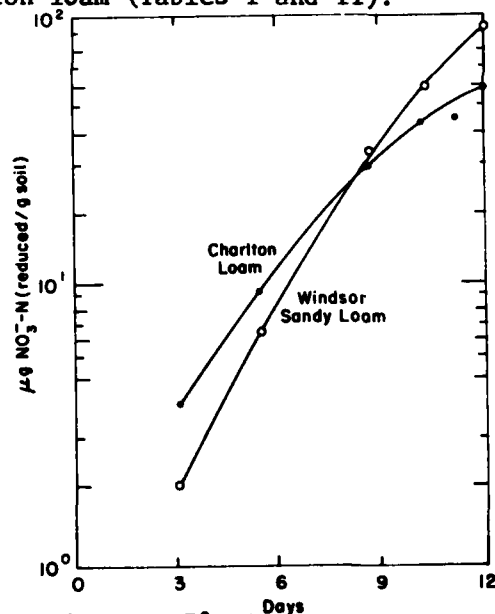than the Charlton loam (Tables I and II).

Figure 6. Nitrate loss at 7°C in the Charlton Loam and the Windsor
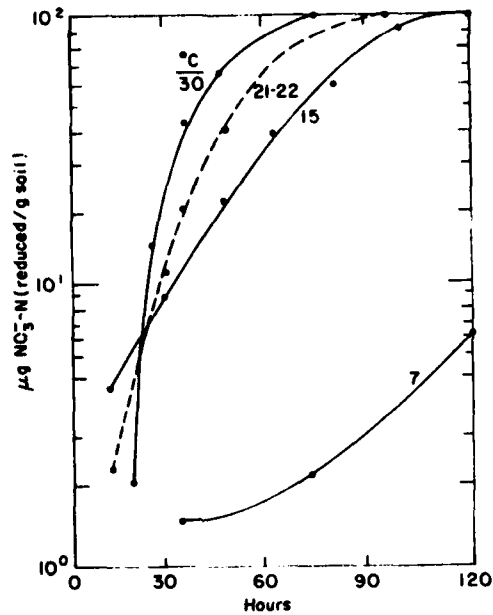sandy loam.

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Figure 7. Nitrate loss in the Windsor sandy loam with glucose as the carbon source at 7°, 15°, room temperature and 30° C.
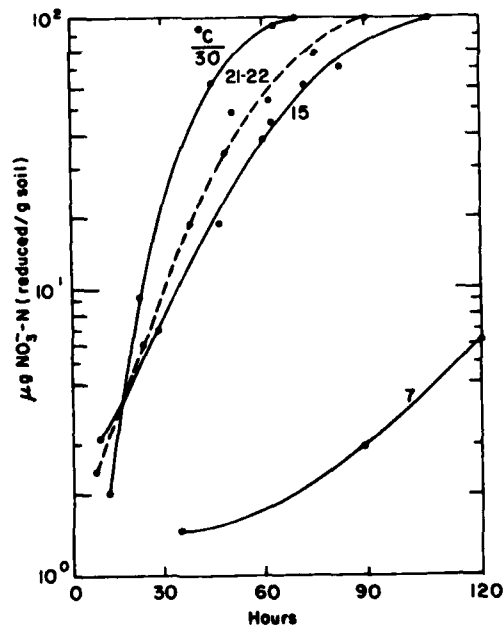

Figure 8. Nitrate loss in the Charlton loam, with glucose at 500 ppm C as the carbon source at 7°, 15°, 21-22°, and 30°C.

Table 1. Kinetics of denitrification in Charlton loam, pH 6.3.

Temp. (°C)	Carbon source	Doubling time for Denitrification (h*)	Apparent lag, (h)
7	Glucose 500 ppm C	50	65
15	Glucose 500 ppm C	14	20
21-22	Glucose 500 ppm C	13	8
21-22	No addition	20	15
30	Glucose 500 ppm C	6.9	6
30	Glucose, 1000 ppm C	6.9	6
30	No addition	17	12
30	Succinate, 500 ppm C	8.5	15
30	Methanol, 500 ppm C	8.4	10
30	Secondary effluent[†]	16	20
30	Secondary effluent**	13	26
30	Sewage effluent[††]	11	21

* During logarithmic phase.
† After chlorination.
** Before chlorination.
†† Before chlorination (9 parts secondary: 1 part primary).

The Q_{10} values for nitrate loss in the Charlton loam in the temperature range of 15-30°C is 1.7. The Q_{10} value for the Windsor sandy loam in the same temperature range is 1.9.

Carbon source effects on denitrification

The effects of various carbon sources on the denitrification rate were determined in the Charlton loam and in the Windsor sandy loam. The Charlton loam samples received glucose at 500 ppm C, glucose at 1000 ppm C, succinate at 500 ppm C, methanol at 500 ppm C, secondary effluent after chlorination, secondary effluent before chlorination, and a blend of 10% primary effluent in unchlorinated secondary effluent (the amounts of effluents added were to bring the soil to field capacity). The rates of nitrate loss for the treated and untreated samples are shown in Table I. The kinetics of nitrate loss in the untreated Charlton loam and in samples that received glucose, succinate and methanol as carbon

Table II. Kinetics of denitrification in several soils and sewage.

System	Temp. (°C)	Carbon source	Doubling time for denitrification (h)	Apparent lag, (h)
Windsor sandy loam, pH 5.5	7	Glucose 500 ppm C	42	65
	15	Glucose 500 ppm C	13	20
	21-22	Glucose 500 ppm C	10	14
	21-22	No addition	17	16
	24	Glucose 500 ppm C	8.9	16
	30	Glucose 500 ppm C	5.5	18
	30	Glucose 1000 ppm C	4.9	16
	30	No addition	13	12
	30	Secondary effluent[†]	28	37
	30	Secondary effluent ***	24	27
	30	Sewage effluent[††]	12	27
Niagara silt loam, pH 6.8	21-22	Glucose 500 ppm C	5.9	15
	24	Glucose 500 ppm C	4.7	16
	30	Glucose 500 ppm C	2.7	19
Lordstown channery silt loam, pH 4.2	30	Glucose 500 ppm C	15	18
Hudson silty clay loam, pH 6.8	30	Glucose 500 ppm C	7.3	9
Sewage primary effluent, pH 7.4	30	Sewage O.M.***	9.4	8

*During logarithmic phase.
†After chlorination.
**Before chlorination.
††Before chlorination (9 parts secondary: 1 part primary effluent).
***Endogenous sewage organic matter.

sources is shown in Figure 9, and the effects of the effluents on nitrate loss are shown in Figure 10.

A dramatic way to show the effects of the carbon source on denitrification in the Charlton loam is to compare the percentage change in
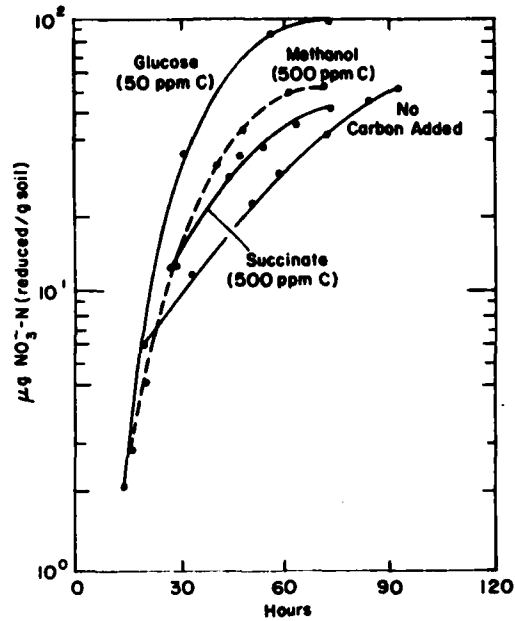
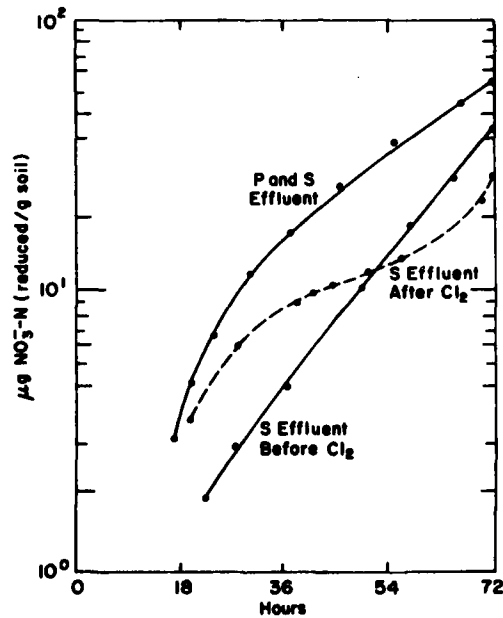Figure 9. Effect of carbon source on nitrate loss in the Charlton loam at 30°C.

Figure 10. Nitrate loss in the Charlton loam at 30° with effluents as carbon source. P means primary, and S means secondary.

the rate of nitrate loss to the rate observed when no carbon source is added (Table III). Here, one can see that secondary effluent, both before and after chlorination, is not very useful in stimulating denitrification. The effluent mixture is more useful, though not as rich as methanol, succinate and glucose.

Table III. Effect of carbon source on the denitrification rate in Charlton loam.

Temp. (°C)	Carbon source	Decrease in doubling time for denitrification (%)*
21-22	Glucose 500 ppm C	35
30	Glucose 500 ppm C	59
30	Glucose 1000 ppm C	59
30	Succinate 500 ppm C	50
30	Methanol 500 ppm C	51
30	Secondary effluent[†]	5.8
30	Secondary effluent **	24
30	Sewage effluent[††]	35

*Compared to the rate observed during logarithmic phase when no carbon source is added.

†After chlorination.
**Before chlorination.
††Before chlorination (9 parts secondary: 1 part primary effluent).

The Windsor sandy loam samples received, in addition to glucose at 500 and 1000 ppm C, secondary effluent both before and after chlorination, and 10% primary effluent in unchlorinated secondary effluent (effluents were added to bring the soil to field capacity). The rates of nitrate loss in the Windsor sandy loam with the different carbon sources (and with no carbon source) are given in Table II, and the effects of the carbon sources on the kinetics of nitrate loss are shown in Figures 11 and 12.

Table IV shows the percentage changes in the rate of nitrate loss in the Windsor sandy loam with the various carbon sources. In this soil, there is a more marked effect of both the glucose and of the effluents on the denitrification rate than in the Charlton loam. The
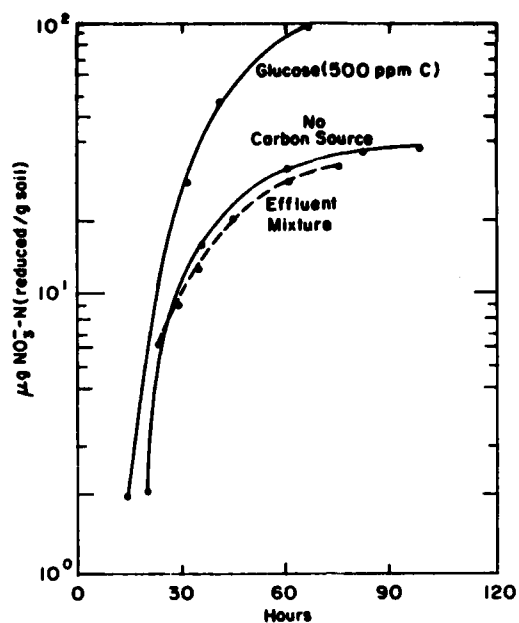
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Figure 11.   Effect of carbon source on rate of nitrate loss in the
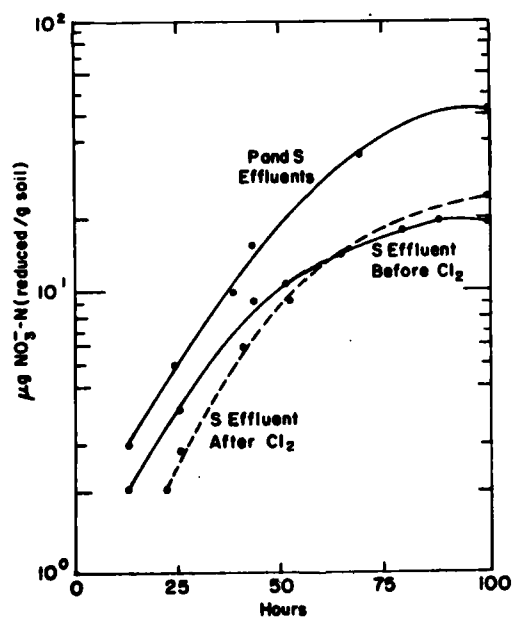Windsor sandy loam at 30°.

Figure 12.   Effect of effluents on nitrate loss in the Windsor sandy
loam at 30°.   P means primary, and S means secondary.

Table IV. Effect of carbon source on the denitrification rate in
           Windsor sandy loam.

Temp. (°C)	Carbon source	Decrease in doubling time for denitrification* (%)
21-22	Glucose 500 ppm C	41
30	Glucose 500 ppm C	58
30	Glucose 1000 ppm C	62
30	Secondary effluent[†]	-120
30	Secondary effluent**	- 69
30	Sewage effluent[††]	7.7

*Compared to the rate observed during the logarithmic phase
 when no carbon source is added.

†After chlorination.

**Before chlorination.
††Before chlorination (9 parts secondary: 1 part primary effluent).

addition of glucose increased the denitrification rate by 58% compared
to the rate observed in unamended soil. The chlorinated secondary
effluent actually reduced the denitrification rate by 120%, as compared
to unamended soil, and surprisingly, the unchlorinated secondary effluent
also reduced the reaction rate. The rate of increase in denitrification
rate resulting from the application of the effluent mixture was only
7.7%.

Denitrification rate in different soils

The denitrification rate in five different soils was studied at
30°C. Glucose at 500 ppm was the carbon source. The denitrification
rates for the five soils are shown in Tables I and II. In the Lordstown
channery silt loam with a pH of 4.2, the denitrification rate (expressed
as the doubling time for nitrate loss during the logarithmic phase of
nitrate loss) was low (15 h). In soils with a pH near neutrality, the
rate of nitrate loss was higher. This is shown in the observed doubling
time for the rate of nitrate loss in Windsor sandy loam (pH 5.5)
(5.5 h), Charlton loam (pH 6.3) (6.9 h), Hudson silty clay loam (pH
6.8) (7.5 h), and Niagara silt loam (pH 6.8) (2.7 h).

Nitrate accumulation and loss

As part of the determination of the denitrification activities, the accumulation and loss of nitrite was observed in the nitrate-amended soils. In the Lordstown channery silt loam, no nitrite was observed to accumulate at 30°C with glucose added. The absence of nitrite in the Lordstown channery silt loam was anticipated because of the instability of nitrite at low pH values. No nitrite was also observed in the other forest soil, the Niagara silt loam, at 21-22°, 24° and 30°C. The lack of nitrite accumulating in the Niagara silt loam was surprising because of its pH of 6.8.

In the Charlton loam, there was an observable accumulation and subsequent loss of nitrite. At 30°C, with glucose as a carbon source, nitrite accumulated to over 30% of the initial added nitrate concentration (Fig. 13) after about 2 days. The accumulation of nitrite was followed by its rapid disappearance. At lower temperatures and with other carbon sources than glucose (Fig. 14), the nitrite accumulation was not as rapid or as great. The other carbon sources led to later accumulation of nitrite, with smaller amounts being accumulated but with similar times for nitrite loss. Lower temperatures also produced a slower nitrite accumulation with lesser amounts being accumulated and longer time periods being required for elimination of the nitrite.
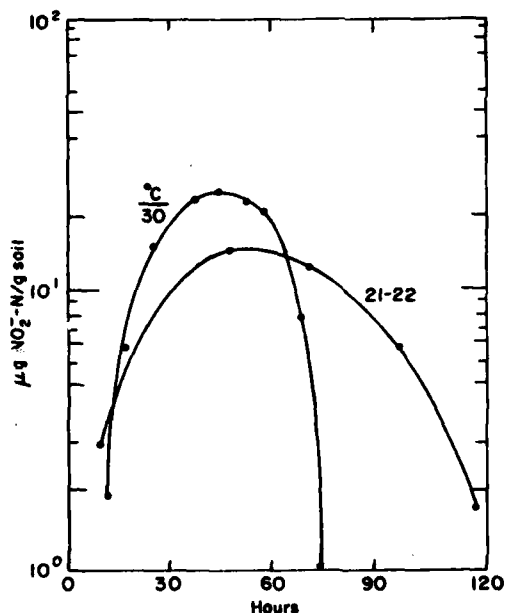

Figure 13. Nitrite accumulation and loss in the Charlton loam, at 30°C and at room temperature (21-22°C). Glucose at 500 ppm C was the carbon source.

Table V. Kinetics of denitrification at 30°C in soils treated with inhibitors.

Soil	Inhibitor	Doubling time for denitrification, (h*)	Apparent lag, (h)
Charlton loam, pH 6.3	Chlorate		
	10 ppm	8.8	12
	50 ppm	13	24
	100 ppm	8.0	30
	500 ppm	– ***	–
	Streptomycin		
	2500 ppm	7.0	6.6
	Tetracycline		
	100 ppm	9.0	22
	250 ppm	9.0	22
	500 ppm	10	37
	1000 ppm	10	54
	Penicillin[†]		
	100 ppm	7.0	9.0
	500 ppm	14	34
	1000 ppm	15	47
	2000 ppm	14	55
	5000 ppm	– ***	–
	No inhibitor	6.9	7.0
Lordstown channery silt loam, pH 4.2	Actidione, penicillin**	15	18
	Actidione, streptomycin**	30	36
	Penicillin, streptomycin**	– ***	–
	No inhibitors	15	18

* During logarithmic phase
†Penicillin activity, 1667 units/mg.
**Each antibiotic at 1000 ppm.
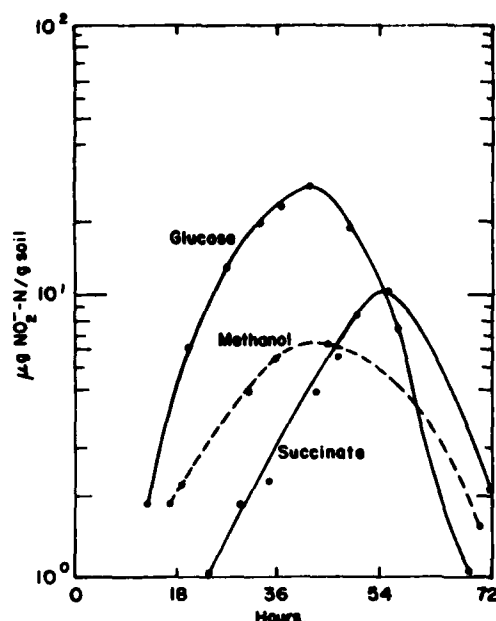***Doubling time too long to measure.

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Figure 14. Effect of carbon source on nitrite accumulation in the
Charlton loam at 30°C. All treatments received 500 ppm
C.

Nitrite accumulation and loss in the Windsor sandy loam also
showed a pattern (Fig. 15) similar to that seen in the Charlton loam.
In the Windsor sandy loam, the nitrite concentration only reached about
25% of the initial added nitrate concentration at 30°C when glucose
was added.

Inhibitor effects on denitrification

To determine the nature of the active denitrifier populations
in the Charlton loam and the Lordstown channery silt loam, the rates
of nitrate loss and nitrite accumulation and loss in those two soils
were carried out at 30°C with glucose at 500 ppm C as the carbon source,
but with the addition of metabolic inhibitors. The inhibitory compounds
were added with the thought that, if the inhibition of denitrification
could be achieved by a chemical that selectively inhibits certain types
of bacteria, this would then offer an indication as to which organisms
are responsible for denitrification in that soil.

The Charlton loam received chlorate, a mimic of nitrate and a
competitive inhibitor of nitrate reductase (42,43) and streptomycin,
tetracycline, and penicillin, three inhibitors of bacterial growth.
The Lordstown channery silt loam received penicillin plus actidione
(an inhibitor of fungal growth) streptomycin plus actidione and penicillin
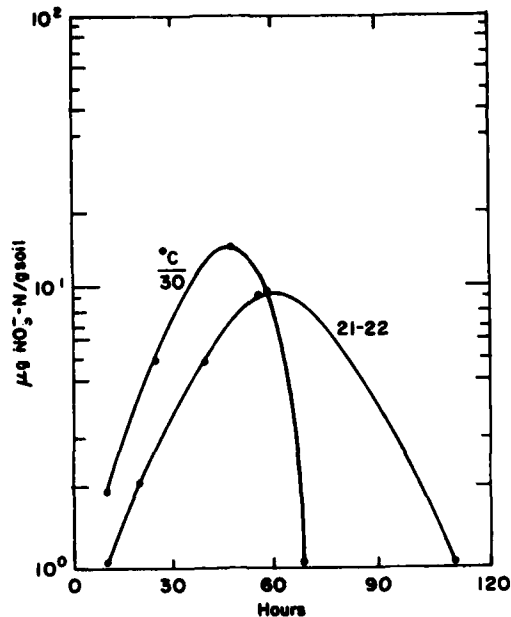plus streptomycin.

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Figure 15. Nitrite accumulation in the Windsor sandy loam at 30°C
and at room temperature. Glucose at 500 ppm C is the
carbon source.

Chlorate is a potent inhibitor of denitrification in the Charlton
loam (Table V, Fig. 16). At 10 ppm chlorate-Cl, the denitrification
rate was inhibited 28%. At a concentration of 50 ppm chlorate-Cl,
there was an initial inhibition of denitrification followed by what
appears to be a resurgence in activity indicating that the chlorate
may have been inactivated. At a chlorate level of 100 ppm Cl, there
was some initial nitrate loss followed by a complete inhibition of
activity. With 500 ppm chlorate-Cl added, there was minimal denitrifi-
cation.

Streptomycin at a high concentration of 2500 ppm led to no in-
hibition of denitrification in the Charlton loam (Table V). Tetracycline
caused some inhibition of denitrification, at least by causing longer
lag periods in the Charlton loam (Table V, Fig. 17). Once the tetra-
cycline-induced lag phase was over, the degree of inhibition of nitrate
reduction was not related to the concentration of tetracycline added.
The longer lag periods caused by the addition of increasingly higher
concentrations of tetracycline and the similarity in subsequent rates
would indicate that bacteria different from the ones originally dominant
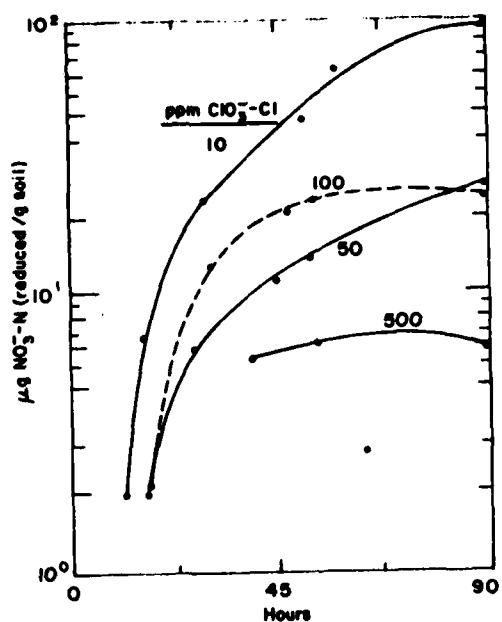had become the new dominant denitrifiers.

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Figure 16. Nitrate loss in the Charlton loam at 30°C treated with
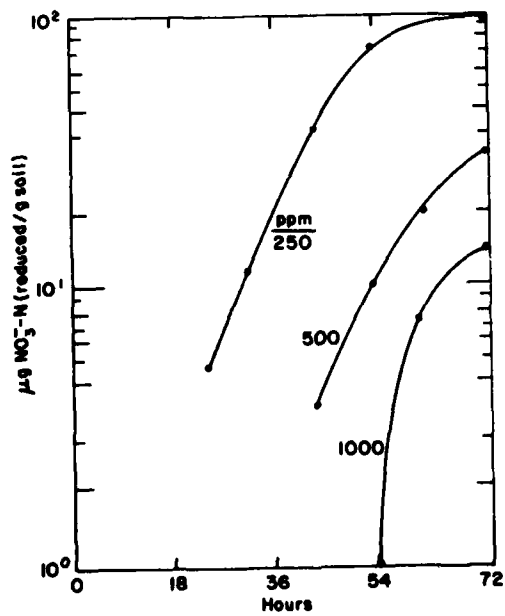KClO₃. Glucose at 500 ppm C was the carbon source.

Figure 17. Nitrate loss in the Charlton loam at 30°C treated with
tetracycline. Glucose at 500 ppm C was the carbon source.

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Penicillin at a low concentration of 100 ppm showed a rapid rate of denitrification in the Charlton loam (Fig. 18). At much higher concentrations of the inhibitor, the duration of lag phase increased (Table V). With 500 ppm of penicillin added, the rate of nitrate reduction was logarithmic. At penicillin concentrations of 1000 and 2000 ppm, the denitrification rate declined markedly after the losses of 30% and 15%, respectively of the nitrate added, indicating that there may not have been enough carbon available to whichever bacteria had become the dominant denitrifiers in the soil at that time. Denitrification ceased completely at a penicillin concentration of 5000 ppm, a very high concentration of inhibitor.
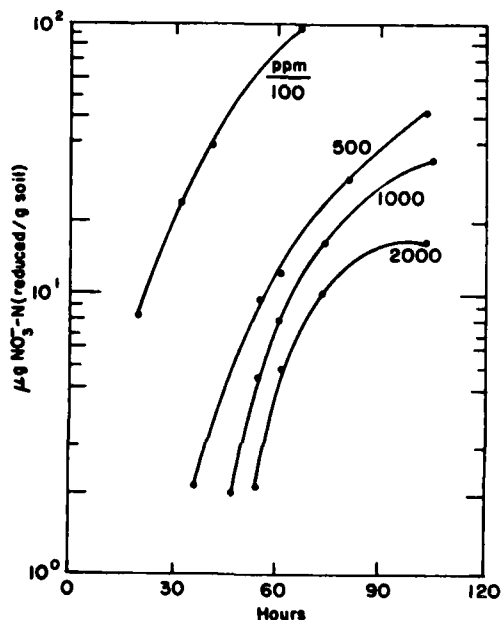

Figure 18. Nitrate loss in the Charlton loam at 30°C treated with penicillin. Glucose at 500 ppm C was the carbon source.

In the Lordstown channery silt loam, actidione was included with the other inhibitors in order to determine if fungi, whose presence in acid soils is more prominent than in neutral and alkaline soils, played a part in the denitrification in this acid soil. The results show that the inhibition of denitrification in the Lordstown channery silt loam was dependent on the presence of streptomycin but not actidione (Fig. 19). The results also show that streptomycin, in conjunction with another antibacterial agent, penicillin, gave the most effective inhibition of denitrification in the Lordstown channery silt loam.
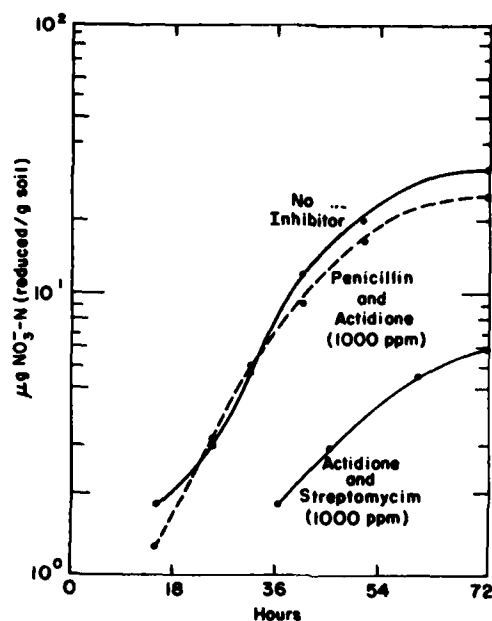
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Figure 19. Nitrate loss in the Lordstown channery silt loam at 30°C
with glucose at 500 ppm C as the carbon source.

Because of the high concentrations of inhibitors involved, the
large numbers of variables and the seemingly equivocal results, the
experiments seeking to identify the active denitrifiers using anti-
biotics and toxicants were terminated and a different approach was
embarked upon.

Comparison of media to count and isolate denitrifiers

Three media, modified casinate agar, soil extract agar, and Difco
nitrate agar, were compared as to their ability to support anaerobic
microbial growth. Spread plates made from the 10^{-5} and 10^{-6} dilutions
of the same soil sample (Charlton loam) were incubated anaerobically.
From 1 g of soil, 1.1×10^6 cells were counted on the casinate agar,
4.3×10^{-5} cells were observed to grow on the soil extract agar, and
2.2×10^7 cells were counted on the Difco nitrate agar. Because of the
higher counts appearing on the nitrate agar, this medium was selected
for use in the denitrifier isolation experiments, and Focht and
Joseph's (34) method of counting denitrifiers utilizing Difco nitrate
broth was chosen to count denitrifiers in soil and sewage.

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Denitrification and denitrifier counts

In order to help assess the relevance and accuracy of the methods for the counting of denitrifiers in soil and to help anticipate denitrifier growth in response to nitrate loss during denitrification, experiments were undertaken to determine the cell growth per unit of nitrate reduced during denitrification. Four denitrifiers, P. stutzeri, P. fluorescens, Pseudomonas sp. and Flavobacterium sp., all soil isolates, were grown anaerobically with nitrate in enriched media (Difco nitrate broth). Values for nitrate lost during denitrification in pure culture range from 0.82 to 2.0 pg NO_3^--N per cell growth (Table VI) and are in accord with earlier reports.

Having determined the expected number of cells per unit of nitrate lost during denitrification, the denitrifier populations during denitrification in soil and sewage were then counted. The counts were made in the Charlton loam, Windsor sandy loam, Hudson silty clay loam, Niagara salt loam and in primary effluent. The growth in cell number per unit of nitrate lost during the logarithmic phase of denitrification in these systems was used to obtain the values for nitrate-N reduced per cell growth.

In the Charlton loam, the denitrifier counts were made during denitrification at 30°C with no glucose added (Fig. 20), at room temperature (21-22°C) (Fig. 21) and at room temperature with glucose added (Fig. 22). In the three cases, the same pattern is evident, that of logarithmic growth as nitrate is actively being taken up, followed by a rapid and marked die-off of cells once the denitrification rate declines. The values for each unit of nitrate-N reduced per cell growth range from 3.6-7.0 pg (Table VI) and are higher than those expected from the pure culture data. The values of nitrate-N lost per cell growth that were observed when no glucose was added to the soil, 6.0 pg at 30° and 7.0 pg at room temperature, were especially higher than expected.

In Windsor sandy loam, denitrifier counts were made during denitrification with glucose added and without an exogenous carbon source at 30°C and at 21-22°C (Table VI, Fig. 23-25). Again, one sees the rapid logarithmic increases in denitrifier populations as the nitrate is disappearing in the same manner.

The entrance of the rate of nitrate loss into the stationary phase again coincides with the dying of many of the denitrifiers. The values for nitrate-N reduced per cell ranged from 4.3 to 8.4 pg and are, as in the Charlton loam, much higher than expected.

Denitrifier counts were also made during denitrification in the Hudson silty clay loam, Niagara silt loam (Fig. 26) and in sewage (Fig. 27). The soils received glucose, and all three systems were incubated at 30"C. The results (Table VI) continue to show that the

Table VI. Nitrate reduced per denitrifier growth.

System*	Temp (°C)	Carbon Source	NO_3^--N reduced per cell (pg)	No. cells $\times 10^5$/μg NO_3^--N
Soils:				
Charlton loam	30	Soil O.M.[†]	6.0	1.6
	21-22	Glucose**	3.6	2.8
	21-22	Soil O.M.	7.0	1.4
Windsor sandy loam	30	Soil O.M.	8.4	1.2
	30	Glucose	4.3	2.3
	21-22	Soil O.M.	7.0	1.4
	21-22	Glucose	7.0	1.4
Hudson silty clay loam	30	Glucose	1.8	5.5
Niagara silt loam	21-22	Glucose	2.0	5.0
Primary effluent	30	Sewage O.M.[††]	6.7	1.5
Pure culture:				
P. stutzeri	30	N.B.***	2.0	5.0
P. fluorescens	30	N.B.	0.82	12
Pseudomonas sp.	30	N.B.	1.5	6.7
Flavobacterium sp.	30	N.B.	2.0	5.0

* For the soils and sewage, the values for NO_3 reduced per cell represent the ratio of the quantity of nitrate reduced divided by the numbers of denitrifiers determined by most-probable-number counts. For the cultures, the numbers of denitrifiers were determined by plate counts.

† Endogenous soil organic matter.

** Glucose at 500 ppm C.

†† Endogenous sewage organic matter.
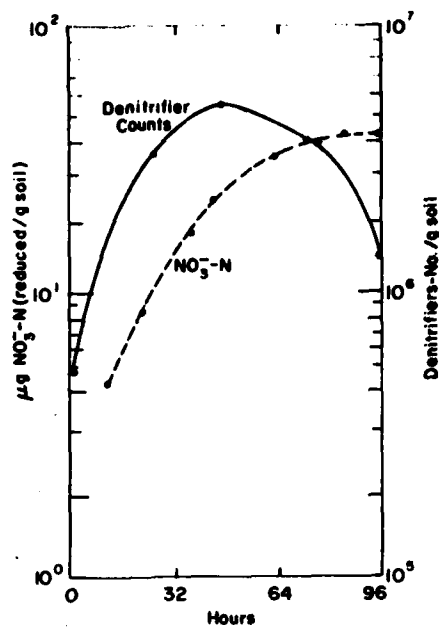
*** Nitrate broth.

Figure 20. Nitrate loss and denitrifier populations in the Charlton loam at 30°C with no glucose added.
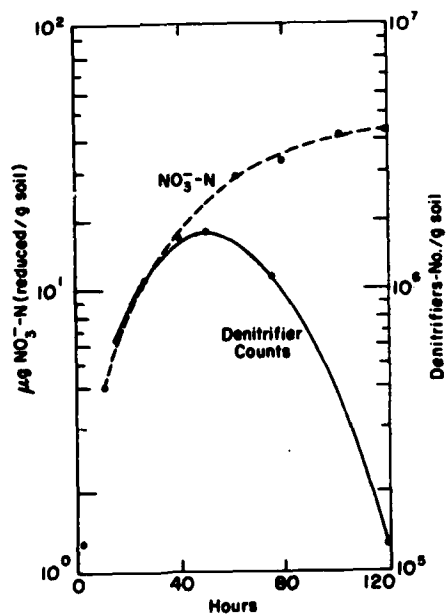

Figure 21. Nitrate loss and denitrifier populations in the Charlton loam at room temperature (21-22°C). No glucose added.
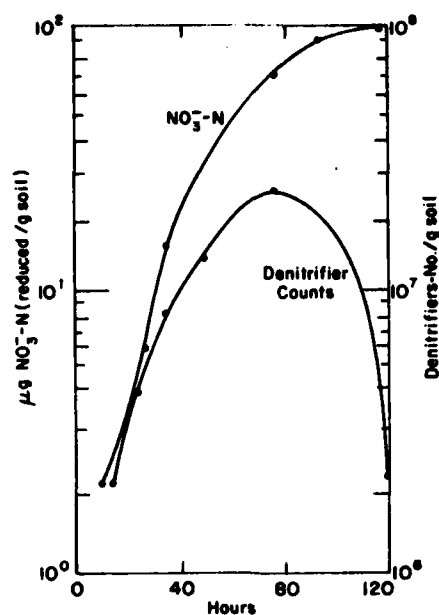
Figure 22. Nitrate loss and denitrified populations in the Charlton loam at room temperature 21-22°C with glucose at 500 ppm C added.

Figure 23. Nitrate loss and denitrifier population in Windsor sandy loam with glucose at 500 ppm C added at 21-22°C.

Figure 24. Nitrate loss and denitrifier growth in the Windsor
sandy loam at 30°C with no glucose added.

Figure 25. Nitrate loss and denitrifier population in Windsor
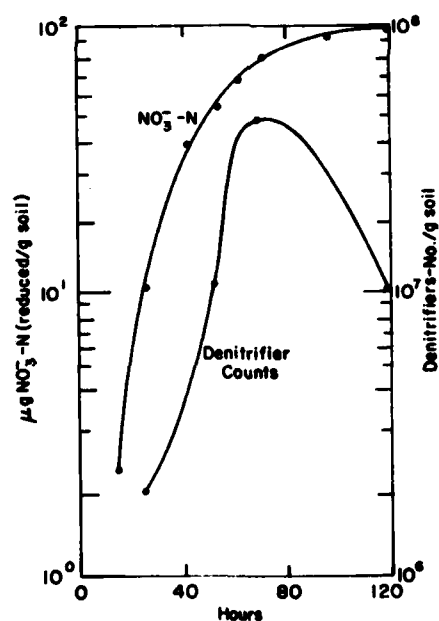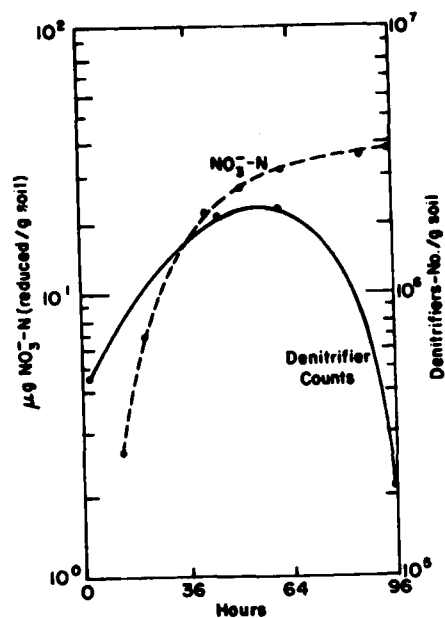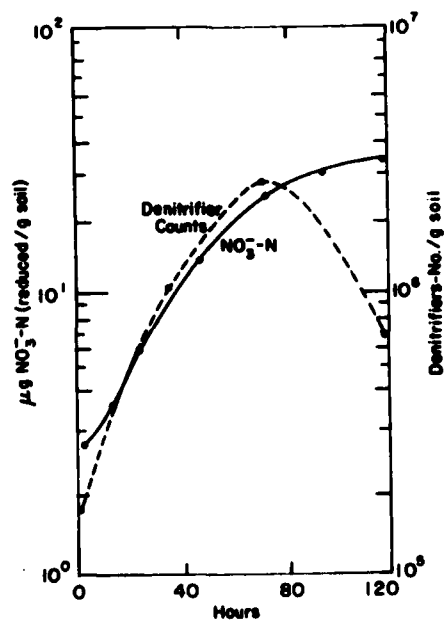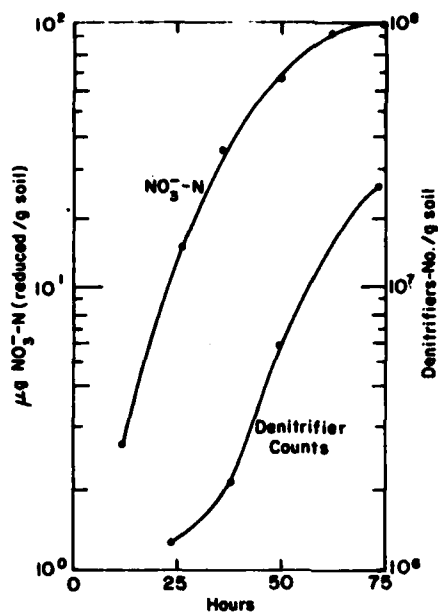sandy loam. No glucose added (21-22°C).

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Figure 26. Nitrate loss and denitrifier growth in the Niagara
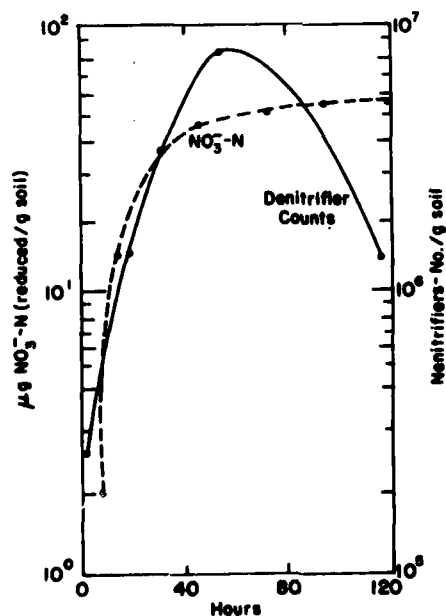silt loam at 22°C. Glucose at 500 ppm C was the
carbon source.

Figure 27. Nitrate loss and denitrifier population in primary
effluent at 30°C.

typical pattern of a dramatic cell die-off occurs when nitrate loss
ceases. The amounts of nitrate-N lost per denitrifying cell are 1.8
pg in the Hudson soil, 2.0 pg in the Niagara soil and 6.7 pg in the
sewage.

Nitrosamine formation during denitrification

Several experiments were performed to determine whether dimethyl-
nitrosamine is formed during denitrification in soil. The Charlton
loam and the Windsor sandy loam were incubated at 24°C in the standard
system used to measure denitrification. The soils were enriched with
100 ppm nitrate-N and 7.0 ppm dimethylamine-N. The Charlton loam re-
ceived glucose solution (500 ppm C), and the Windsor sandy loam received
unchlorinated seccndary effluent to bring the soils to field capacity.
To the system, gas traps containing distilled water were added to trap
any of the dimethylnitrosamine that may have been formed and volatilized.
Soil samples taken during the logarithmic and stationary phases of
nitrate loss and the water in the gas traps were analyzed for the
presence of dimethylnitrosamine. The studies were run for 96 hours and
samples were taken at 0, 24, 36, 48 and 96 h. Though a nitrite level of
28 ppm nitrite-N was seen in the Charlton loam, and an extraordinarily
high nitrite level of 58 ppm nitrite-N was seen in Windsor sandy loam,
no dimethylnitrosamine was detected in the samples that were analyzed.

Determining the active denitrifiers in soil

Experiments were conducted to identify the bacteria which are
responsible for denitrification in a soil. Freshly dug, undried samples
of Hudson silty clay loam were given two separate treatments prior to
anaerobic incubation at 30°C. One set of samples was amended with
glucose and KNO_3 to final concentrations of 500 ppm C and 100 ppm N.
The other set of samples was given the same amount of glucose plus
$(NH_4)_2SO_4$ to a final concentration of 100 ppm N. Glucose was selected
as the carbon source because, in previous experiments, it was the best
stimulator of denitrification. The rate of nitrate loss was noted in
the samples given nitrate, both per gram of soil (Table II) and per
denitrifier cell (Table VI), and the denitrifiers were counted in both
sets of samples (Fig. 28). The denitrification rate was similar to that
in the Charlton loam. The cell number appearing per unit of nitrate-N
lost was the lowest of all of the soils tested, indicating that the
counting methods gave fairly reliable results in this soil.

At zero hours, 100 bacterial colonies were isolated from three
spread plates inoculated with samples from the 10^{-6} dilution of the soil
and incubated anaerobically. Twelve of these colonies were denitrifiers
and were identified as: six Bacillus cereus-subtilis-lichiniformis
types (which were not further identified), four colonies of Alcaligenes
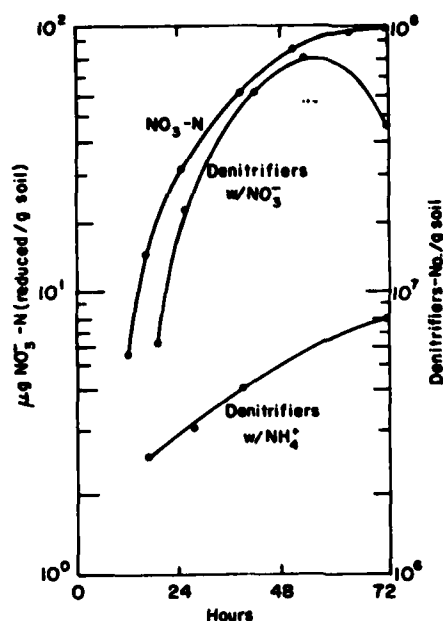
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Figure 28. Nitrate reduction and denitrifier populations in the Hudson silty clay loam treated with glucose as 500 ppm C plus nitrate or ammonium (30°C).

faecalis, and two isolates of Pseudomonas stutzeri/aeruginosa. After 15 h of incubation, 100 colonies were isolated in the same manner from the plates of the 10^{-7} dilution of the nitrate-treated soil. Ten colonies were denitrifiers and were identified as: one B. cereus-subtilis-lichiniformis type, seven isolates of A. facecalis, and two P. aeruginosa. From the ammonium treated soil, 100 colonies were picked from the plates of the 10^{-6} dilution. Twenty-one of the colonies were denitrifiers and were identified as: 14 B. cereus-subtilis-lichiniformis types and seven A. faecalis. After 27 h, 100 colonies were picked from plates of the 10^{-7} dilution of each soil treatment. From the nitrate-treated soil, 11 denitrifiers were found among the 100 isolates, and these were identified as: four B. cereus-subtilis-lichiniformis types, two A. faecalis, four P. stutzeri/aeruginosa, and one Paracoccus denitrificans. From the ammonium-treated soil, eight denitrifiers were found and identified as: six B. cereus-subtilis-lichiniformis types, one B. megaterium, and one A. faecalis.

The last set of isolations were made after 39 h of incubation. Again, 100 isolates were taken from spread plates of the 10^{-7} dilution of each soil treatment. From the nitrate-treated soil, 17 denitrifiers were found and identified as: four B. cereus-subtilis-lichiniformis types, seven A. faecalis, five P. stutzeri/aeruginosa, and one Listeria denitrificans. From the ammonium-treated soil, 10 denitrifiers were

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among the 100 colonies, and identified as: eight B. cereus-subtilis-lichinformis types, and two A. faecalis.

DISCUSSION

The organisms that inhabit a soil are determined by, and in turn have a profound effect on, the environmental factors therein. This makes each soil a unique and dynamic entity with its own microflora and its own set of responses to any and every physical and chemical change. This uniqueness makes it difficult to generalize about the effects of environmental conditions on processes like denitrification, which are mediated by more than one type of bacteria (2,12).

The low denitrification rate in the Lordstown channery silt loam was expected. Although denitrification has been reported at pH values as low as 3.5 (60), Valera and Alexander (78) have shown that there is a positive correlation between the number of denitrifiers in a soil and its pH, with very low numbers present when the pH is below 5.0. They found the denitrifiers to be more acid-sensitive than the other bacteria in the soil.

In the soils with a pH of higher than 5.0 (Windsor sandy loam, Charlton loam, Niagara silt loam, and the Hudson silty clay loam), the pH effect on the denitrifying bacteria was unimportant. Dawson and Murphy (23) found that the optimum pH for denitrification in soil was 7.0. They saw a 50% decrease in the denitrification rates at pH 6.0 and 8.0. Other studies (30) have demonstrated linear increases in the denitrification rate as the pH increased from 4.0, with a leveling of the rate between pH 7.0 and 8.0. On the other hand, Focht and Verstraete (35) predicted that the effect of pH changes (within the range of 5.0-9.0) on the denitrifiers in soil would be minimal. Valera and Alexander (78) showed that the pH effect on denitrifiers in pure culture leveled off at values approaching 6.0, meaning that the pH effect became secondary to other factors in determining denitrification rate.

The observed Q_{10} values of 1.9 for the Windsor sandy loam and 1.7 for the Charlton loam for the doubling time of the rate of nitrate denitrification are consistent with other reported Q_{10} values. These Q_{10} values range from 1.4-3.6 (33). Factors such as substrate diversity and concentration have the greatest effect on the Q_{10} value for denitrification in a soil (35), and the effects of change in temperature on the denitrification rates will vary from soil to soil.

The absence of denitrifying activity at 1°C is in conflict with the speculation of Smid and Beauchamp (72). After performing kinetic studies of denitrification in soil at temperatures of 30°, 15°, 10°, and 5°C, they predicted that denitrification could occur at or very

close to 0°C. Other studies have stated that the denitrification of nitrate may occur below 5°C (9,33).

In the Charlton loam, higher denitrification rates were seen with glucose, methanol or succinate than with sewage effluents or endogenous soil organic matter as a carbon source. In the Windsor sandy loam, glucose also stimulated denitrification more than effluents or native soil organic matter. The denitrification rate in the Niagara silt loam was greater with glucose as the carbon source than with just the soil organic matter. These observations point to the fact that denitrification is dependent on the amount of readily available carbon. Glucose, methanol and succinate are more available carbon sources than are sewage effluents or native soil organic matter. Correlations between the rates of denitrification and the amount of available carbon (19) or even soluble carbon (74) have been reported, while others have reported denitrification to be stimulated by a vast array of organic compounds (28,33).

The lack of an appreciably greater denitrification rate in the Charlton loam and the Windsor sandy loam when 1000 ppm glucose-C was added in place of 500 ppm glucose-C is important. It means that the denitrifiers' need for carbon had been satisfied at additions closer to the 500 ppm C concentration.

In the reaction

$$5C_6H_{12}O_6 + 24NO_3^- \rightarrow 30CO_2 + 18H_2O + 24OH^- + 12N_2 \quad (45)$$
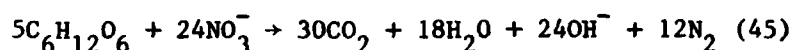
1.07 units of glucose-C should be oxidized for every unit of nitrate-N reduced. Theoretically, the denitrifiers should require 107 ppm of glucose-C when given 100 ppm NO_3^--N. Others (23,35) have reported carbon:nitrogen ratios optimal for denitrification in soil and wastewaters to be in the range of 2-3:1. It is difficult to determine how much (and in what form) the added and/or native carbon is being utilized by the denitrifiers (21). Many other organisms will be competing for the carbon and the nitrate.

The extent of nitrate loss was also affected by the carbon source. Glucose at 500 ppm C or at 1000 ppm C was the only carbon source that stimulated complete nitrate removal from the soils. In the Charlton loam, the addition of methanol and succinate led only to the reduction of 70% and 50% of the added nitrate, while adding glucose led to total nitrate loss after the same time period. Adding sewage effluents or not adding any carbon to soils brought about an incomplete loss of nitrate. Primary effluent itself was not a rich enough carbon source to support complete nitrate loss.

Several studies (21,33) have demonstrated methanol to be the cheapest and most efficient carbon source for denitrification. Others (53) have reported primary effluent mixed with secondary effluent to be an effective carbon source in removing nitrate from sewage. Though methanol and primary effluent are less expensive than glucose they have not been seen to effect as rapid or complete removal of nitrate during denitrification as glucose. Primary effluent is not as available a carbon source as glucose.

Glucose can be fermented by soil bacteria to a wide variety of energy-rich compounds. For this reason, glucose is a richer and more available energy source to the soil microflora than methanol or succinate. With repeated applications of any energy source to soil, a denitrifier population will be selected for that which will make the energy source a readily usable and effective stimulator for denitrification in that soil.

The Charlton loam and the Windsor sandy loam were investigated with the eventual aim of use by the U.S. Army Corps of Engineers as media for the removal of nitrogen from wastewater. The results of the experiments comparing the denitrification rates in these soils show that the Windsor sandy loam would cost more to use than the Charlton loam. With the carbon source being the sewage effluents, the Charlton loam showed quicker denitrification rates than the Windsor sandy loam. The Windsor sandy loam showed quicker rates than the Charlton loam when glucose, a more expensive carbon source than sewage effluents, was added. A wastewater treatment program using the Windsor sandy loam would require the utilization of an exogenous carbon compound like glucose or methanol, and such additions would constitute an added expense to the program. In the Charlton loam, by contrast, the sewage effluent mixture is certainly the most useful carbon source for stimulating denitrification because it does not require an added expense.

The addition of selective inhibitors to demonstrate the contribution of the various microbial groups to soil reactions was first done in the Soviet Union by microbiologists who attempted to inhibit denitrification (39). Later, Anderson and Domsch (3) added streptomycin and actidione to soils to determine the relative bacterial and fungal contributions to soil respiration. In the present study, the Charlton loam received chlorate, an anion that is a competitive inhibitor of nitrate reductase (42,43,51), or the antibiotics, penicillin, streptomycin and tetracycline, which inhibit the growth of prokaryatic organisms (17,54). Streptomycin had no inhibitory effect on the denitrifying activity in the Charlton loam. Chlorate, penicillin and tetracycline appear to have been at first very effective against the denitrifiers by increasing the lag time for the reduction of nitrate. Chlorate, which may have been reduced to chlorite, another inhibitor of nitrate reduction (66), remained an effective inhibitor of denitrification. Penicillin

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and tetracycline both failed to completely suppress denitrification as
evidenced by the logarithmic rate of nitrate loss after the prolonged
lag phase. These two antibiotics could have been inactivated by ad-
sorption to soil constituents, or by chemical or biological decomposition
(1), or they could have selected for a different denitrifying flora.

The Lordstown channery silt loam received, in three separate treat-
ments, actidione, a fungal inhibitor (16) with penicillin, actidione
with streptomycin, and streptomycin with penicillin. Actidione with
penicillin caused no inhibition of denitrifying activity. The failure
of actidione to cause an inhibition of nitrate reduction would rule
out a role for fungi in denitrification in the Lordstown channery silt
loam. Adding streptomycin, in place of penicillin along with actidione,
slightly inhibited denitrification, while streptomycin with penicillin
totally suppressed denitrification. These results would indicate that
the dominant denitrifiers were sensitive to streptomycin. The synergistic
effect of penicillin and streptomycin together on denitrification in
the Lordstown channery silt loam suggests that the bacteria that suc-
ceeded the streptomycin-sensitive denitrifiers were sensitive to
penicillin.

The experiments in which inhibitors were added to the soils prior
to the onset of denitrification were performed to help determine the
dominant denitrifiers in those soils. The results were too equivocal
and ambiguous to justify the pursuing of this approach towards the
identification of the active and dominant denitrifiers in the soils.

Values for nitrate lost per cell grown during denitrification in
pure cultures of four denitrifiers were determined. The values obtained
using P. stutzeri, P. fluorescens, Pseudomonas sp., and Flavobacterium
sp. of 2.0, 0.82, 1.5, and 2.0 pg of NO_3-N lost per cell, respectively,
are similar to other observed values (29,49,50,79,80,86). Verhoeven
(79,80), using P. aeruginosa, found there to be 1.4 pg of NO_3-N lost
per cell. Workers in Japan (49,50) found there to be a loss of 0.5
pg of NO_3-N per Pseudomonas denitrificans cell. The losses of nitrate
per denitrifying cell in pure culture are helpful in predicting and
assessing denitrification activity in soil, sewage or water.

The nitrate loss per denitrifier cell in soil and sewage was
determined. In the environments with a complex carbon source for
denitrification, like the organic components of sewage or unamended
soil, there is a higher than expected loss of nitrate-N per cell, the
values ranging from 6.7 to 8.4 pg. In the soils receiving glucose,
a more readily available carbon source, all but one of the figures
for nitrate-N loss per cell range from 1.8 to 4.3 pg, which is con-
siderably closer to the anticipated results. A possible explanation
for this phenomenon is that the denitrifier-counting medium used is
selective and supports the denitrifiers which would be most likely to

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utilize simple organic compounds such as sugars or their fermentation products. The medium used to count denitrifiers, Difco nitrate both, was compared to two other media and yielded higher counts from the same soil sample. Also, nitrate broth has been called the most reliable medium by other researchers (34,85). Nonetheless, there are a significant number of denitrifiers in the soil and sewage whose requirements for anaerobic growth are not being met in the nitrate broth. These cells are not appearing in the counts, but they are in the soil because of the very large amount of nitrate disappearing in relation to the number of cells being counted.

Also, in the soil and the sewage, there are other factors that would give the higher values for nitrate reduced per denitrifier cell. In the first place, soil and sewage are not pure cultures. Many organisms (both denitrifiers and nondenitrifiers) will be competing for and using the nitrate so that some of the nitrate will be taken up by nitrate assimilators. Second, the carbon sources in the sewage and unamended soil will both be more complex and less plentiful than in the culture medium. There will be both slower growth in the sewage and in the soil and less efficient nitrate utilization. Third, sewage and soil are not as rich in available cofactors and micronutrients as in nitrate broth. These factors will contribute to a lower cell yield per unit of nitrate lost during denitrification.

Nitrite accumulated in the effluent and in all but two of the soils (Lordstown channery silt loam and Niagara silt loam) to quantities that represented a high proportion of the nitrate added before reduction. Nitrite accumulation is expected during denitrification in neutral environments due to several factors. First, nitrate reductase has a quicker turnover rate of substrate than nitrite reductase (44). Second, there are many organisms in the soil that will perform dissimilatory nitrate reduction, reducing the nitrate only to nitrite. The number of these organisms may approach or exceed (37) the numbers of denitrifiers in the soil. These two factors will contribute to the nitrite buildup.

The two forest soils, Lordstown channery silt loam and Niagara silt loam, showed no nitrite accumulation. This was anticipated in the case of the Lordstown soil, because it has a pH of 4.2 and nitrite is known to decompose chemically in highly acid soils (2). It is also possible that nitrite never accumulated and was biologically reduced. The chemical decomposition of nitrite was not expected in the Niagara silt loam where the pH is 6.8. The nitrate lost per denitrifier cell in the Niagara silt loam is 2.0 pg NO_3-N. This value is consistent with the values derived from studies of pure cultures of denitrifiers, indicating that the nitrite was biologically reduced.

Nitrosamines have been found to be formed in samples of soil (7,56), water, and sewage (6) to which an amine and large amounts of nitrite have been added. Since nitrite may appear and accumulate during denitrification, it was considered useful to investigate nitrosamine formation during denitrification.

In several experiments, dimethylamine was added to soil prior to the onset of denitrification to determine if dimethylnitrosamine, a carcinogen, might be formed from the amine and from the nitrite produced during denitrification. No dimethylnitrosamine was found. The lack of dimethylnitrosamine accumulation may have resulted from the fact that dimethylamine had disappeared before an appreciable amount of nitrite had accumulated.

The possibility of nitrosamines being formed resulting from nitrite formation in soil still exists. Further experiments should be undertaken to investigate nitrosamine formation in the soil utilizing other amines and looking at nitrosamine formation during nitrification.

Most of the laboratory research on denitrifiers has been performed on _Paracoccus denitrificans_, _Pseudomonas denitrificans_ and _Pseudomonas perfectomarinus_ (37), and while research on these organisms has led to understanding of denitrification at the cellular level, little is known about which organisms are the major denitrifiers in nature.

Denitrification, a process brought about by many types of bacteria, can happen in most environments and is important in wastewater treatment. These characteristics of denitrification make the identification of the active denitrifiers in the Hudson silty clay loam important. Knowing which bacteria are the active denitrifiers in the Hudson silty clay loam lends greater understanding to the overall process in nature, and aids in predicting nitrogen fluctuation in this soil. In addition, a knowledge of the identity and characteristics of the active denitrifiers would assist the operators of a wastewater treatment project in determining the loading rates for obtaining the optimum nitrogen removal capabilities of the system. In the Hudson silty clay loam, there are two distinct groups of denitrifiers. At zero time and in all of the ammonium-treated soil samples, the dominant denitrifying bacteria are the gram-positive spore formers. These gram-positive bacilli, though present, appear to have a small role in the denitrifying activity of the soil when nitrate and glucose are added and are passive denitrifiers. The gram-negative denitrifiers, especially _A. faecalis_, and, to a lesser degree, those bacteria in the genus _Pseudomonas_, are the active denitrifiers in the Hudson silty clay loam, as is shown by their ascendance to numerical dominance once denitrification has begun.

The findings that the gram-negative Alcaligenes and Pseudomonas types of bacteria are the active denitrifiers in the Hudson silty clay loam are consistent with the work of others (35). Gamble et al. (37) found P. fluorescens and Alcaligenes types of bacteria to be the dominant denitrifiers in soil, lake sediment and oxidized poultry manure samples. Alexander (1) mentions the active denitrifiers as belonging to the genera Pseudomonas, Bacillus and Paracoccus. Valera and Alexander (78), and Vives and Pares (81) have found the dominant denitrifiers in soil samples to be those bacteria in the Alcaligenes and Pseudomonas group. Nommik (60) and Woldendorp (91) have shown that organisms in the genus Bacillus may be active in denitrification in some soils. Yet, Vives and Pares (83) found only one Bacillus isolate in the several dozen soil samples examined. The present finding of Paracoccus denitrificans and Listeria denitrificans in the Hudson silty clay loam, while not totally expected, is not surprising, since these denitrifiers are in-digenous to soil (18,22).

The rise in the number of denitrifiers in the ammonium-treated soil was not unexpected. A small rise in denitrifiers was anticipated because of the denitrification of trace amounts of NO_3^- initially present in the soil. Also, the denitrifiers could have benefited from the nitrification of some of the ammonium present, which was probably oxidized at least to nitrite with the trace levels of O_2 that were left in the soil after the purging with N_2 of the flasks holding the soil. The remaining O_2 could also have served as an electron acceptor for the denitrifiers and caused a slight increase in their numbers.

The significance of this study is that it helps to predict the rates of nitrate removal from wastewater and soil. Knowledge of the effects of temperature, pH, and carbon source on denitrification, the degree of nitrite accumulation during denitrification, and the amount of nitrate lost per cell will be useful to the designer of a wastewater treatment program in maximizing the efficiency of such a program. The identifying of the active denitrifiers in a soil is of importance because it confers a greater understanding of the process in nature. The ambiguous results of the inhibitor experiments are pertinent because they illustrate the dynamic nature of the process of denitrification and bacterial succession in soil.

SUMMARY

The influence of soil pH, carbon source and temperature on denitrification in soils was studied. The growth response of denitrifiers in relation to nitrate reduced in soil, sewage and in pure culture was determined. Also, the bacteria responsible for denitrification in a soil were identified.

Denitrification proceeded slowly in a strongly acid soil (pH 4.2). In soils with higher pH values (5.5-6.8), the denitrification rate was faster, and no correlation between the denitrification rate and the pH was observed.

Glucose supported the fastest denitrification rate of all the carbon sources tested. Methanol and succinate gave rates 75% of that of glucose. The rate of nitrate loss was much slower with secondary effluents as the source of supplemental carbon than with glucose, methanol or succinate. Mixing primary effluent with the secondary effluent (9 parts secondary effluent: 1 part primary effluent) slightly increased the denitrification rate compared to the rate observed when only secondary effluent was added, but the effluent mixture was not as good an energy source for the reduction of nitrate as methanol, succinate or glucose.

Charlton loam (pH 6.3) and Windsor sandy loam (pH 5.5) were studied extensively for their denitrification capacities. The Windsor sandy loam showed a more rapid denitrification rate with glucose as the carbon source than the Charlton loam, but the latter soil supported the higher rate of denitrification when sewage was the carbon source. The Q_{10} values for denitrification in the Windsor sandy loam and in the Charlton loam were 1.9 and 1.7. No denitrification occurred in either soil at 1°C. At temperatures of 7°, 15°, 21-22° and 30°C, the doubling time for nitrate loss during the logarithmic phase of denitrification in Charlton loam was 50, 14, 13 and 6.9 h. At temperatures of 7°, 15°, 21-22°, 24° and 30°C, the doubling times for nitrate loss during the logarithmic phase of denitrification in the Windsor sandy loam were 42, 12, 10, 8.9 and 49 h, respectively.

In a study of four denitrifiers (Pseudomonas stutzeri, Pseudomonas fluorescens, Pseudomonas sp. and Flavobacterium sp.) in culture, 2.0, 0.82, 1.5 and 2.0 pg of nitrate-N were destroyed per cell during the logarithmic phase of denitrification. In soils, from 1.8 to 8.4 pg of nitrate-N were lost per countable denitrifier cell, and in primary effluent, 6.7 pg nitrate-N were lost per cell. Lower values of nitrate loss per cell (1.8-4.3 pg) were observed in soils receiving glucose.

No dimethylnitrosamine was detected during denitrification in the Windsor sandy loam and the Charlton loam despite the addition of dimethylamine, though the possibility still exists that nitrosamines may be formed in soil during denitrification.

Experiments were carried out to identify the active denitrifiers in soil. The denitrifier populations during active denitrification in Hudson silty clay loam were noted. The active denitrifiers were identified as <u>Alcaligenes</u> <u>faecalis</u>, <u>Pseudomonas</u> <u>stutzeri</u> and <u>Pseudomonas</u> <u>aeruginosa</u>.

This study provides information on denitrification kinetics in soils in relation to carbon source, pH, temperature, and denitrifier cell growth. In addition, the active denitrifiers in a soil were identified. The data presented here contribute to an understanding of the process of denitrification in nature.

LITERATURE CITED

1. Alexander, M. (1977) Introduction to soil microbiology. 2nd edition. New York: John Wiley and Sons.

2. Alexander, M. (1971) Microbial ecology. New York: John Wiley and Sons.

3. Anderson, J.P.E., and K.H. Domsch (1975) Measurement of bacterial and fungal contributions to respiration of selected agricultural and forest soils. Canadian Journal of Microbiology, vol. 21, p. 314-322.

4. Anthonisen, A.C., T.B.S. Prakasam, and E.G. Smith (1976) Inhibition of nitrification by ammonia and nitrous acid. Soil Science Society of America Proceedings, vol. 35, p. 68-71.

5. Ardakani, M.S., L.W. Belser, and A.D. McLaren (1975) Reduction of nitrate in a soil column during continuous flow. Soil Science Society of America Proceedings, vol. 39, p. 290-294.

6. Ayanaba, A., and M. Alexander (1974) Transformations of methylamine and formation of a hazardous product dimethylnitrosamine in samples of treated sewage and lake water. Journal of Environmental Quality, vol. 3, p. 83-89.

7. Ayanaba, A., W. Verstrate, and M. Alexander (1973) Formation of dimethylnitrosamine in soils treated with nitrogen compounds. Soil Science Society of America Proceedings, vol. 37, p. 564-568.

8. Bailey, L.D. (1971) Effects of temperature and root on denitrification in soil. Canadian Journal of Soil Science, vol. 56, p. 79-87.

9. Bailey, L.D., and E.G. Beauchamp (1973) Effects of temperature on NO_3^- and NO_2^- reduction, nitrogenous gas production and redox potential in a saturated soil. Canadian Journal of Soil Science, vol. 53, p. 213-218.

10. Barker, A.V. (1974) Nitrate determinations in soil, water and plants. Massachusetts Agricultural Experiment Station Research Bull. 611.

11. Birch, N.F. (1958) The effect of drying on humus decomposition and nitrogen availability. Plant Soil, vol. 10, p. 9-31.

12. Bollag, J.-M., M.L. Oreutt, and B. Bollag (1970) Denitrification by isolated soil bacteria under various environmental conditions. Soil Science Society of America Proceedings, vol. 34, p. 875-879.

13. Bollag, J.-M., and G. Tung (1972) Nitrous oxide release by soil fungi. _Soil Biology and Biochemistry_, vol. 4, p. 271-276.

14. Bouwer, H., J.C. Lance, and M.S. Riggs (1974) High rate land treatment II: Water quality and economic aspects of the Flushing Meadows project. _Journal of Water Pollution Control Federation_, vol. 4, p. 844-859.

15. Brady, N.C. (1974) The nature and property of soils. 8th edition, New York: Macmillan.

16. Bremner, J.M., and K. Shaw (1958) Denitrification in soil. II. Factors affecting denitrification. _Journal of Agricultural Science_, vol. 51, p. 40-52.

17. Brock, T.P. (1974) Biology of microorganisms. 2nd edition. Englewood Cliffs, New Jersey: Prentice-Hall, Inc.

18. Buchanan, R.E., and N.E. Gibbons (ed.) (1974) Bergey's manual of determinative bacteriology, 8th edition. Baltimore: Williams and Wilkins.

19. Burford, J.R., and J.M. Bremner (1975) Relationship between the denitrification capacity of soils and total water soluble and readily decomposable soil or organic matter. _Soil Biology and Biochemistry_, vol. 7, p. 389-411.

20. Cady, F.B. and W.V. Bartholomew (1960) Sequential products of anaerobic nitrification in Norfolk soil. _Soil Science Society of America Proceedings_, vol. 24, p. 477-482.

21. Davenport, L.A., W.P. Lembke, and B.A. Jones (1975) Denitrification in laboratory sand columns. _Transactions of the American Society of Agricultural Engineers_, vol. 18, p. 95-99.

22. Davis, D.H., R.Y. Stanier, M. Doudoroff, and M. Mandel (1970) Taxonomic studies on some gram negative polarly flagellated "hydrogen bacteria" and related species. _Archive für Mikrobiologie_ vol. 70, p. 1-13.

23. Dawson, R.W., and K.L. Murphy (1973) Factors affecting the biological denitrification of wastewater, p. 671-680. In (S.N. Jenkins, Ed), _Advances in water pollution research_, Oxford: Pergamon Press.

24. Dawson, R.W. and K.L. Murphy (1972) The temperature dependence of biological denitrification. _Water Research_, vol. 6, p. 71-83.

25. Deherain, P.O. (1897) La réduction des nitrates dans la terre arable. Compte Rendus Academy of Sciences Paris, vol. 124, p. 269-273.

26. Deibel, R.H., and J.B. Evans (1960) Modified benzidine test for the detection of cytochrome containing respiratory systems in microorganisms. Journal of Bacteriology, vol. 79, p. 356-360.

27. Delwiche, C.C., and B.A. Bryan (1976) Denitrification. Annual Review of Microbiology, vol. 30, p. 241-263.

28. Doner, H.E. (1975) Disappearance of nitrate under transient conditions in columns of soil. Soil Biology and Biochemistry, vol. 7, p. 257-259.

29. Doner, H.E., M.G. Volz, L.W. Belser, and J.P. Løken (1975) Short term nitrate losses and associated microbial populations in soil columns. Soil Biology and Biochemistry, vol. 7, p. 261-265.

30. Ekpete, D.M., and A.M. Cornfield (1965) Effect of pH and addition of organic matter on denitrification losses from soil. Nature, vol. 208, p. 1200.

31. Engel, M.S., and M. Alexander (1958) Growth and autotrophic metabolism of Nitrosomonas europaea. Journal of Bacteriology, vol. 76, p. 217-222.

32. Etinger-Tulczynska, R. (1969) A comparative study of nitrification in soils from arid and semiarid areas of Israel. Journal of Soil Science, vol. 20, p. 307-317.

33. Focht, D.D., and A.C. Chang (1975) Nitrification and denitrification processes related to wastewater treatment. Advances in Applied Microbiology, vol. 19, p. 153-186.

34. Focht, D.D., and H. Joseph (1973) An improved method for the enumeration of denitrifying bacteria. Soil Science Society of America Proceedings, vol. 37, p. 698-699.

35. Focht, D.D., and W. Verstrate (1977) Biochemical ecology of nitrification and denitrification. Advances in Microbial Ecology, vol. 1, p. 135-214.

36. Fred, E.B. and S.A. Waksman (1928) Laboratory manual of general microbiology. New York: McGraw-Hill.

37. Gamble, T.N., M.R. Betlach, and J.M. Tiedje (1977) Numerically dominant denitrifying bacteria from world soils. _Applied Environmental Microbiology_, vol. 33, p. 926-939.

38. Gayon, V. and G. Dupetit (1886) Réduction des nitrates par les infiniments petits. Mem. Soc. Bordeaux Serie 3, vol. 2, p. 200-204 (Cited by Focht and Verstrate. 1977.

39. Geller, I.A., K.M. Dobrotvorskaya, and V.P. Karpenko (1960) Application of antibiotics for partial inhibition of denitrification processes (in Russian). _Agrokhimiya_, vol 4, p. 111-115 (Cited by Focht and Verstrate. 1977).

40. Goering, J.J., and J.D. Cline (1970) A note on denitrification in sea water. _Limnology and Oceanography_, vol. 15, p. 306-308.

41. Gordon, R.E., W.C. Haynes and C.H.N. Pang (1973) The Genus _Bacillus_. U.S. Department of Agriculture Handbook no. 427, Washington.

42. Hackenthal, E. (1966) Die parollele Induktion von Nitratreduktion von Nitritreduktion bei _Bacillus_ _cereus_ durch verschiedene Anionon. _Biochemical Pharmacology_. 15:1119-1126. (Cited in: Payne, W.J. 1973) Reduction of nitrogenous oxides by microorganisms. _Bacteriology Review_, vol. 37, p. 409-452.

43. Hackenthal, E., and D. Arbazadeh (1966) Kompetitive Inhibitoren der Nitrat Reduktion in Zelfrein Extrakten aus _Bacillus_ _ceveus_ und _Pseudomonas_ _aeruginosa_. _Zeifschrift for Physiological Chemistry_, vol. 344, p. 180-188. (Cited in: Payne, W.J. 1973).

44. Haddock, B.N., and C.W. Jones (1977) Bacterial respiration. _Bacteriology Review_, vol. 41, p. 47-99.

45. Hardy, R.W.F., and R.D. Holsten (1972) Gobal nitrogen cycling: Pools, evolution, transformations, transfers, quantitation and research needs. In _The aquatic environment: Microbial transformations and water management implications_ (L.J. Guarria and R.K. Ballentine eds.), p. 87-132.

46. Hauck, R.D., and S.W. Molsted (1956) Some aspects of the problem of evaluating denitrification in soils. _Soil Science Society of America Proceedings_, vol. 20, p. 361-364.

47. Johnston, H. (1972) Newly recognized vital nitrogen cycle. _Proceedings of the National Academy of Sciences U.S.A._, vol. 69, p. 2369-2372.

48. Knuth, D.T. (1970) Nitrogen cycle ecology of solid waste composting. Compost Science, vol. 11, p. 8-12.

49. Koike, I., and A. Hattori (1975) Growth yield of a denitrifying bacterium Pseudomonas denitrificans under aerobiosis and anaerobiosis. Journal of General Microbiology, vol. 88, p. 1-10.

50. Koike, I., and A. Hattori (1975) Energy yield of denitrification: An estimate from growth yield in continuous cultures of Pseudomonas denitrificans under limiting conditions. Journal of General Microbiology, vol. 88, p. 11-19.

51. Korki, N., P. Kaiser, and J. Ponchon (1972) Effets du chlorate de sodium sur les microbes de la dénitrification dans le sol. Étude écologique à laboratoire. Comptes Rendus, Academy of Sciences, Paris, vol. 274, p. 2809-2814.

52. Labeda, D.P., R.S. Wodzinski and M. Alexander (1977) Effect of sulfur dioxide on nitrification in soil. Abstract, Annual Meeting of American Society of Microbiologists, p. 232.

53. Lance, J.C., and F.D. Whisler (1976) Stimulation of denitrification by adding organic carbon to wastewater. Journal of the Water Pollution Control Federation, vol. 48, p. 346-356.

54. Lenninger, A.C. (1970) Biochemistry. New York: Worth Co.

55. Mclaren, A.D. (1976) Rate constants for nitrification and denitrification in soils. Radiation and Environmental Biophysics, vol. 13, p. 43-48.

56. Mills, A.L. (1976) Nitrosation of secondary amines by axenic cultures of microorganisms and in samples of natural ecosystems. Ph.D. Thesis (unpublished), Cornell University.

57. Morrill, L.G., and J.E. Dawson (1967) Patterns observed for the oxidation of ammonium to nitrate by soil organisms. Soil Science Society of America Proceedings, vol. 31, p. 757-760.

58. Mulbarger, M.C. (1971) Nitrification and denitrification in activated sludge systems. Journal of the Water Pollution Control Federation, vol. 46, p. 1984-1994.

59. Murray, I., J.W. Parsons, and K. Robinson (1975) Interrelationships between nitrogen balance, pH, and dissolved oxygen in an oxidation ditch treating farm animal waste. Water Research, vol. 9, p. 25-30.

60. Nömmik, H. (1956) Investigations on denitrification in soil. Acta-Agric. Scand., vol. 6, p. 195-228.

61. Onderdonk, A.B., J. Johnston, J.W. Mayhew, and S.L. Gorbach (1976) Effect of dissolved oxygen and E_h on Bacteroides fragilis during continuous culture. Applied and Environmental Microbiology, vol. 31, p. 168-172.

62. Orion Research (1976) Methods manual, 93 series electrode. Cambridge, Mass.

63. Painter, H.A. (1970) A review of literature of inorganic nitrogen metabolism in microorganisms. Water Research, vol. 4, p. 393-450.

64. Parker, C.A. (1955) Anaerobiosis with iron wool. Australian Journal of Experimental Biology, vol. 33, p. 33-37.

65. Payne, W.J. (1970) Energy yields and growth of heterotrophs. Annual Review of Microbiology, vol. 24, p. 17-52.

66. Payne, W.J. (1973) Reduction of nitrogenous oxides by microorganisms. Bacteriology Review, vol. 37, p. 409-452.

67. Prakasam, T.B.S., and D.C. Loehr (1972) Microbial nitrification and denitrification in concentrated wastes. Water Research, vol. 6, p. 859-869.

68. Richards, F.A., and W.W. Broenkow (1971) Chemical changes including nitrate reduction in Darwin Bay, Galapagos Archipelago over a 2-month period, 1969. Limnology and Oceanography, vol. 16, p. 758-765.

69. Schloesing, J.J.T., and A. Muntz (1877) Sur la nitrification par les ferments organisés. Compte Rendus, Academy of Sciences, Paris, vol. 84, p. 301-303.

70. Skerman, V.B.D. (1967) A guide to the identification of the genera of bacteria. Baltimore: Williams and Wilkins.

71. Skerman, V.B.D., and J.C.MacRae (1957) The influence of oxygen on the reduction of nitrate by adapted cells of Pseudomonas denitrificans. Canadian Journal of Microbiology, vol. 3, p. 215-230.

72. Smid, A.T., and E.G. Beauchamp (1976) Effects of temperature and organic matter on denitrification in soil. Canadian Journal of Soil Science, vol. 56, p. 385-391.

73. Stanford, D.W., S. Dzienia, and R.A. Vander Pol (1975) Effect of temperature on denitrification rate in soils. Soil Science Society of America Proceedings, vol. 39, p. 867-875.

74. Stanford, G., R.A. Vander Pol, and S. Dzienia (1975) Denitrification rates in relation to total and extractable soil carbon. Soil Science Society of America Proceedings, vol. 39, p. 284-289.

75. Starr, J.L., F.E. Broadbent, and D.R. Neilsen (1974) Nitrogen transformations during continuous leaching. Soil Science Society of America Proceedings, vol. 38, p. 283-289.

76. Stefanson, R.C. (1972) Soil denitrification in sealed soil-plant systems. I. Effects of plants, soil-water content and soil organic matter. Plant Soil, vol. 37, p. 113-127.

77. Tate, R.L., and M. Alexander (1976) Resistance of nitrosamines to microbial attack. Journal of Environmental Quality, vol. 5, p. 131-133.

78. Valera, C.R., and M. Alexander (1961) Nutrition and physiology of denitrifying bacteria. Plant Soil, vol. 15, p. 268-280.

79. Verhoeven, W. (1950) On a spore forming bacteria causing the swelling of cans containing cured ham. Antonie van Leeuwenhoek J. Microbiol. Serol, vol. 16, p. 269-281.

80. Verhoeven, W., and J.J.C. Goos (1954) Studies on true dissimilatory nitrate reduction. I. Antonie van Leeuwenhoek J. Microbiol. Serol, vol. 20, p. 93-101.

81. Verstraete, W., and M. Alexander (1972) Heterotrophic nitrification by Arthrobacter sp. J. of Bacteriology, vol. 110, p. 955-961.

82. Verstraete, W., and M. Alexander (1973) Heterotrophic nitrification in samples of natural ecosystems. Environmental Science and Technology, vol. 7, p. 39-42.

83. Vives, J. and R. Pares (1973) Enumeracion y caracterizacion de la flora desnitrificante quimioorganotrosa en una pradera experimental. Microbiol. Espan., vol. 28, p. 43-53.

84. Voets, J.B., H. Vanstaen, and W. Verstrate (1975) Removal of nitrogen from highly nitrogenous wastewaters. Journal of the Water Pollution Control Federation, vol. 47, p. 394-398.

85. Volz, M.G. (1977) Assessing two diagnostic methods for enumeration of nitrate reducing and denitrifying bacteria in soil-plant root associations. *Journal of Soil Science Society of America*, vol. 41, p. 337-340.

86. Volz, M.G., L.W. Belser, M.S. Ardakani, and A.D. McLaren (1975) Nitrate reduction and associated microbial populations in a ponded Hanford sandy loam. *Journal of Environmental Quality*, vol. 4, p. 99-102.

87. Weber, D.F., and P.L. Gainey (1962) Relative sensitivity of nitrifying organisms to hydrogen ions in soils and in solution. *Soil Science*, vol. 94, p. 138-148.

88. Weissenberg, H. (1902) Über die denitrifikation. *Zentrabl. Bakteriol.*, Parasitenk II., Abt. 2, vol. 8, p. 166-170. (Cited in: Focht and Verstrate 1977).

89. Wijler, J. and C.C. Delwiche (1954) Investigations on the denitrifying process in soil. *Plant Soil*, vol. 5, p. 155-169.

90. Winogrodsky, S. (1890) Sur les organismes de la nitrification. *C. R. Academy of Sciences, Paris*. vol. 100, p. 1013-1016.

91. Woldendorp, J.W. (1966) Losses of soil nitrogen. *Stikstof*, vol. 12, p. 32-46.

92. Wong-Chong, C.M., and R.C. Loehr (1973) The kinetics of microbial nitrification. *Water Research*, vol. 9, p. 1099-1106.

93. Wührmann, K. (1964) Microbial aspects of water pollution control. *Advances in Applied Microbiology*, vol. 6, p. 119-151.

94. Yoshida, T., and M. Alexander (1970) Nitrous oxide formation by *Nitrosomonas europaea* and heterotrophic microorganisms. *Soil Science Society of America Proceedings*, vol. 34, p. 880-882.